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Anatomical Features of the Tubercle and Young Sporophyte of the Annual Fern *Anogramma chaerophylla* Growing in the Punta Lara Natural Reserve (Buenos Aires, Argentina)

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Abstract.—The fern genus *Anogramma* is characterized by the presence of annual sporophytes and tubercles that persist through dry periods. Tubercles may host embryos that develop when climatic conditions are more favorable. As information on the structure of the tubercle and sporophyte of *Anogramma chaerophylla* is incomplete, the objective of this work was to analyze anatomical characteristics during development and deepen knowledge of the adaptive strategies of this species. Spores were grown *in-vitro* and different stages of tubercle development and embryonic sporophytes were fixed in FAA, embedded in Paraplast and analyzed using light microscopy. Initially, the green gametophyte developed antheridia and archegonia in its thickened portion, where later bisexual tubercles differentiated. Embryos developed only from the archegonia located in the tubercles, with one embryo developing per tubercule. During sporophyte growth the reserves accumulated in the tubercle were consumed. Oversized cells were observed at the junction between the green gametophyte and the tubercle, suggesting a possible role in translocation of substances. The young sporophyte consisted of a short shoot and a prominent foot. The placenta comprised the foot cells and the adjacent tubercle cells. The first leaves protruded soon and developed early as photosynthetic organs. Sporophytes remained attached to the tubercles until advanced stages of development. Vegetative propagation was documented in smaller tubercles that did not develop gametangia. Because few sporophytes were observed in vivo, it is likely that natural populations are maintained through vegetative propagation of the gametophyte until favorable conditions encourage development of sporophytes.

Key Words.—anatomy, gametophyte, embryo, adaptations, *Anogramma*

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Anogramma Link (Pteridaceae) is a homosporous fern genus with seven species distributed mainly in the tropics of Mexico and Central and South America and less frequently in Africa, from southern Europe to northern India, and in Australia and New Zealand (Tryon *et al.*, 1990; Nakazato and Gastony, 2003). In Argentina, *Anogramma* is represented by two species: *Anogramma chaerophylla* (Desv.) Link and *Anogramma lorentzii* (Hieron.) Diels.

The genus is characterized by the presence of annual sporophytes (1_40 cm height) that often grow in environments with alternating wet and dry seasons. A striking feature of *Anogramma* is that gametophytes are considered perennial because they carry tubercles that can become dormant during stressful periods, or support the expansion of a latent embryo when favorable conditions arrive (Goebel, 1889; Baroutsis, 1976; Mehra and Sandhu, 1976; Page, 2002). Bower (1923) described the tubercles as "archegoniophores" because they carry numerous archegonia.

Most studies on gametophyte development and morphology were conducted in the widely distributed species *Anogramma leptophylla* (L.) Link (Mehra and Sandu, 1976; Pangua and Vega, 1996; Hagemann, 1997). Goebel (1889) described the first stages of gametophyte development for *Anogramma chaerophylla* and mentioned that the tubercles store starch grains and other food reserves. Later, Hagemann (1997) extended study of the gametophyte of *A. chaerophylla* by describing meticulously its development and tubercle formation.

Anogramma chaerophylla is widely distributed in Central and South America, from Mexico, El Salvador and the Greater Antilles to the Galapagos, Brazil, Paraguay, Uruguay, and Argentina (Mickel and Smith, 2004). In Argentina, this species grows from Misiones and Jujuy Provinces in the north, through the center-east of the country, reaching its southernmost distribution in Buenos Aires Province (Zuloaga, Morrone, and Belgrano, 2008).

Because information on tubercle and sporophyte structure and development in *Anogramma chaerophylla* is incomplete, the objective of this work was to analyze the anatomical characteristics of both stages at different times during their formation. This knowledge may contribute to understanding adaptive strategies in ferns.

Materials and Methods

Sampling area.—Fertile fronds were collected in Punta Lara Natural Reserve, Buenos Aires, Argentina (34° 47′ S, 58° 01′ W) spanning three years (2012-2014) from November to April. The Reserve is situated on the Coast of La Plata River where the mean annual temperature is 16.5 °C and the precipitation averages 950 mm per year, with fluctuating periods of flooding and drought. In this habitat, ferns grow mainly associated with the gallery forests that border the internal streams. During hikes in the Reserve in different seasons few plants of *Anogramma chaerophylla* growing in isolation were observed.

Cultivation of spores.—To analyze different stages of tubercle and sporophyte development, spores were grown in vitro. Portions of fronds with mature closed sporangia were placed in paper envelopes until spore release. Then, spores were sown in previously sterilized Petri dishes, 5 cm in diameter containing Murashige-Skoog medium (Murashige and Skoog, 1962) and Difco Bacto-agar (7 g/l). Twenty dishes were prepared and kept under laboratory conditions, with a photoperiod of 12 hours light under white fluorescent illumination 28 μmol m^{-2} s^{-1} at 22–24° C.

Cultivation of gametophytes and sporophytes.—Once gametophytes acquired their typical helical shape, they were transferred to plastic pots with fertile soil and brought outdoors covered by a black shadow cloth. The pots were watered weekly. Different phases of gametophyte and sporophyte development were detected through weekly observations under a stereoscopic microscope (Nikon SMZ 1000).

Anatomical study.—The structure of the gametophyte, tubercle, and sporophyte at different developmental stages was analyzed under a light microscope. Materials were fixed in formaldehyde-acetic acid-alcohol, dehydrated through an ethanol series and embedded in Paraplast. Microtome sections (8-10 μm thick) were double stained with safranin-fast green (Johansen, 1940). Polarized light and Lugol's solution were used to ascertain the presence of starch grains (D'Ambroggio de Argüeso, 1986). Observations and photographs were carried out with a Nikon E200 light microscope.

RESULTS

Green thalloid gametophyte.—The initial filament emerging from the spore divides longitudinally, producing a spatula-shaped structure with a lateral meristem. The spore germination pattern corresponds to the *Vittaria* type and the prothallus development is *Ceratopteris* type (Luna et al., in press). The gametophyte continues to develop asymmetrically, bending itself around the growth zone to acquire a funnel shape (Fig. 1A). The innermost zone has multiple cell layers, which then decrease to only a single-cell layer in thickness along the exterior of the gametophyte (Fig. 1B). Archegonia and antheridia are differentiated in the thickened portion of the gametophyte, together with numerous rhizoids. The neck of the archegonium protrudes from the gametophyte and its diameter is made up by 4 rows of cells and is 4_5 cells in length (Fig. 1C). The antheridium consists of the basal cell, the ring cell, and the cap cell (Fig 1D). Gametangia continue differentiating a certain distance from the multicellular portion. In some instances, fertilization is evidenced by the presence of a zygote and then an embryo (Figs. 1E-G). At this point of gametophyte development tubercles have already originated.

Gametophyte tubercle.—The origin of tubercles is revealed by the appearance of rounded, whitish (non-photosynthetic) structures in the gametophyte meristematic region (Fig. 2A). The surface of the tubercle is covered by abundant rhizoids (Fig. 2B). At this stage of tubercle development, both the green gametophyte and the tubercle contain gametangia (Fig. 2C). Tubercles are bisexual, i.e., archegonia as well as antheridia are developed, the former in increased number (Figs. 2D-F). The venters of the archegonia are

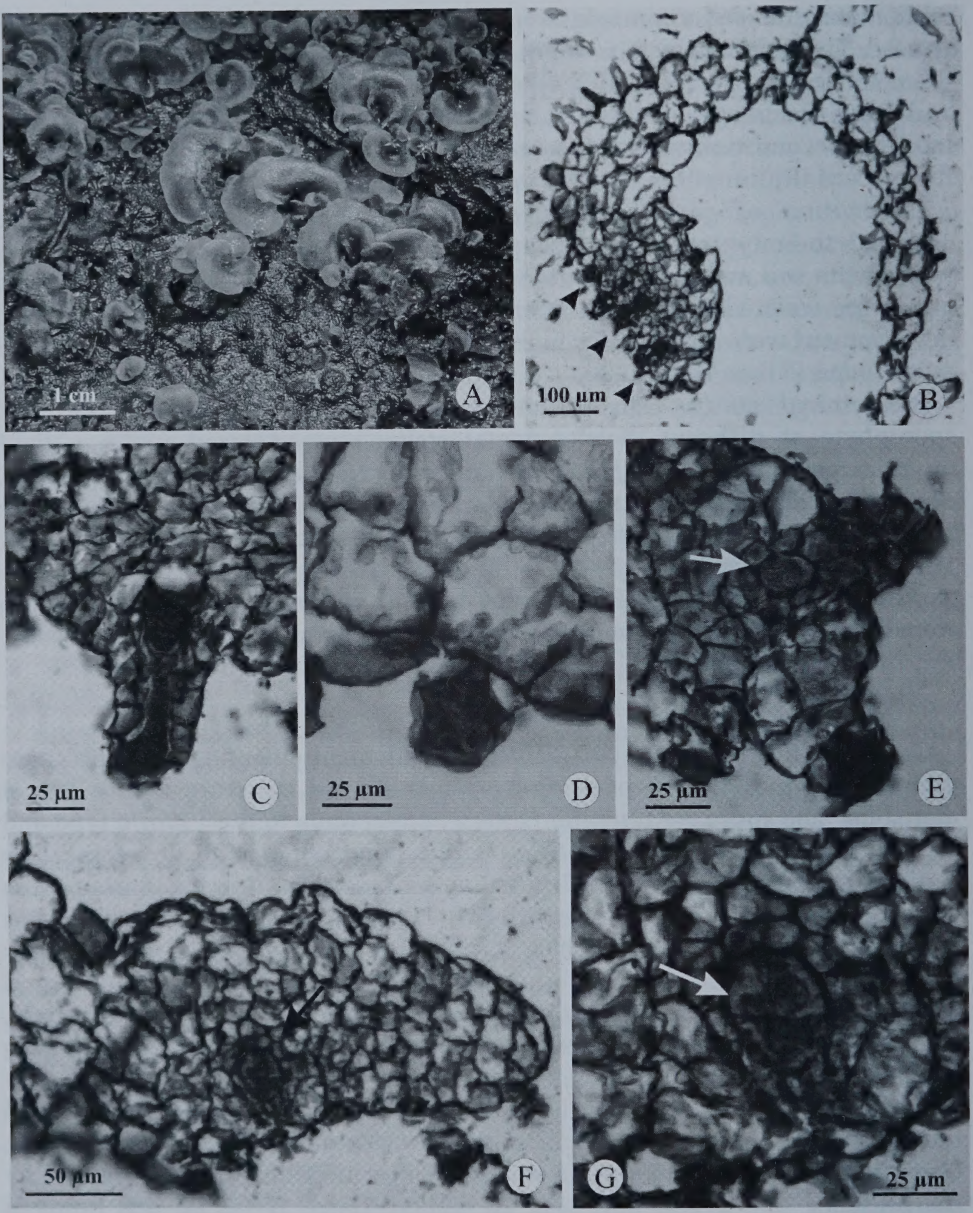

Fig. 1. Morphology of the green gametophyte. A: Macroscopic view of funnel-shaped prothalli. B-G: Light micrographs. B: Section of a green gametophyte showing gametangia (arrowheads) in the multi-layered portion. C: Detail of an archegonium with the neck protruding from the gametophyte. D: An antheridium in detail. E: Fertilized archegonium with a zygote (arrow). F: Archegonium with a developing embryo (arrow). G: Detail of first mitotic divisions of the embryo (arrow).

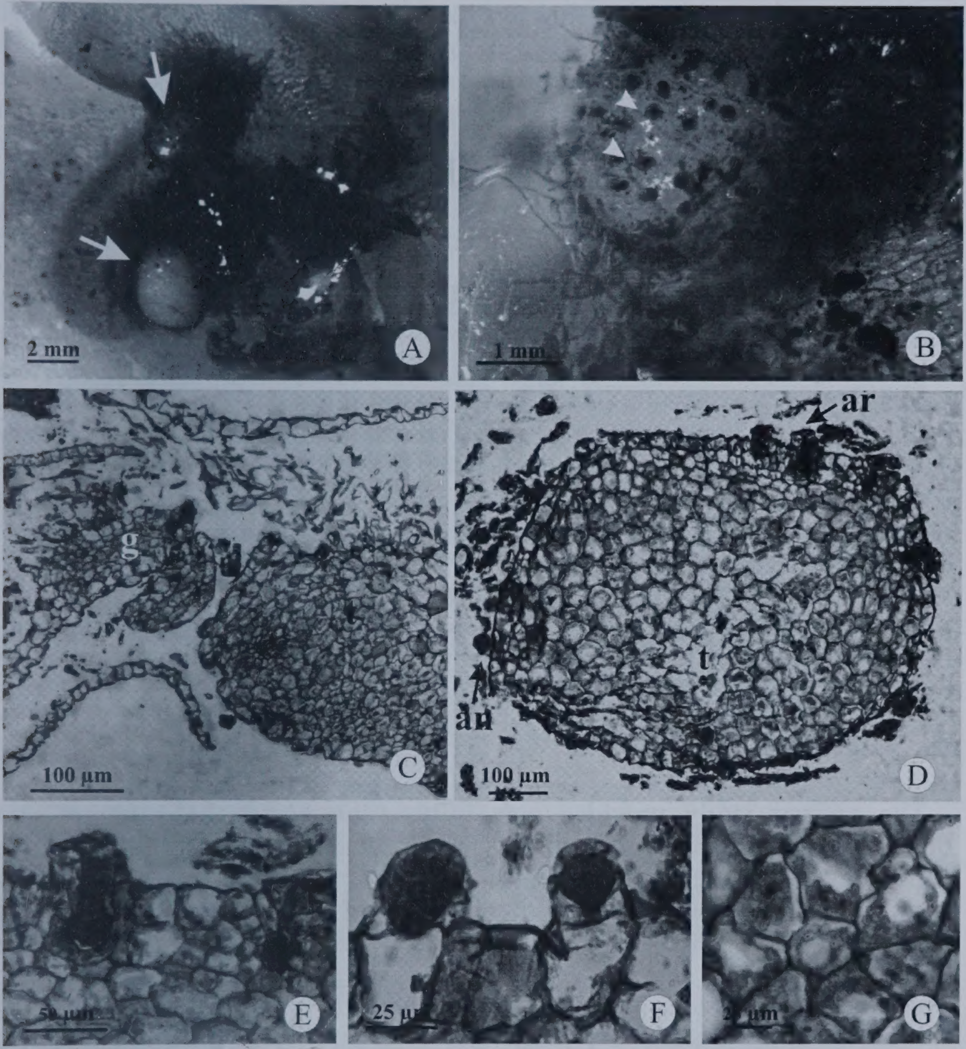

Fig. 2. Morphology of the tubercle. A-B: Macroscopic views. A: General aspect of tubercles covered (arrows) by rhizoids, developed on the green gametophyte' bottom surface. B: Detail of tubercle with numerous archegonia (arrowheads). C-G: Light micrographs. C: Section of a green gametophyte with a developed tubercle (right). Gametangia are observed in both zones. D: Detail of bisexual tubercle. E: Magnification of archegonia with their venters sunk in the tubercle. F: Magnification of antheridia showing basal, annular and opercular cells and antherozoids inside. G: Detail of deposits of starch grains in the tubercle. Abbreviations: antheridium (an), archegonium (ar), green gametophyte (g), tubercle (t)

sunk in the tubercle and their necks protrude 3–4 cells in length (Fig. 2 E). The antheridia show the typical basal, ring, and cap cells, and carry numerous antherozoids (Fig. 2F). The tubercle cells are thin walled and contain starch grains (Fig. 2G). As the tubercle matures, reserves accumulate inside its cells.

Embryo and sporophyte.—After fertilization only one embryo was observed in each tubercle (Fig. 3A). At this point, oversized cells at the junction between the green gametophyte and the tubercle were observed (Fig. 3B). The embryo appears initially globose and then the shoot apex, the foot and the suspensor are differentiated (Figs. 3A, C). As the sporophyte grows, the tubercle opens forming a "calyptralike" outgrowth that allows the emergence of the shoot apex (Fig. 3D). Then the first developing leaves are observed (Fig. 3E). Nearly four months after spore sowing, the sporophyte consists of a short shoot with well-developed vascular tissues and a prominent foot (Fig. 4A). The foot includes cells with dense cytoplasm packed with abundant plastids. The placenta comprises the sporophyte foot cells and the adjacent gametophyte tubercle cells. Interdigitation between the cells of the two generations was noted (Fig. 4B). Intercellular spaces between sporophyte and gametophyte tissues were not observed. The shoot grows little in length and remains attached to the tubercle while the leaves develop (Fig 4C). Roots originate endogenously from the short shoot. During the development of the leaves the reserves of the tubercle are consumed (Fig. 4D). Vegetative propagation is also observed in the form of smaller tubercles that do not develop gametangia (Figs. 4D, E). During sporophyte growth, the first leaf developed early as a photosynthetic organ (Fig. 5A). The other leaves originate successively from the short shoot, while the green gametophyte and the tubercle remain functional in appearance (Fig. 5B).

Discussion

The present study provides new information about the structure and development of the *Anogramma chaerophylla* gametophyte and sporophyte. During green gametophyte growth, we observed the development of antheridia and archegonia in the multilayered zone. Although Hagemann (1997) did not detect archegonia in the green gametophytes of *A. chaerophylla*, we agree with Baroutsis (1976) in that archegonia may be present outside the tubercle. Occasionally, we detected fertilization and embryo development in the archegonia of the green gametophyte; however, in these cases, the sporophyte generation did not survive beyond a few mitotic divisions.

The green gametophyte of *Anogramma chaerophylla* produced bisexual tubercles on its lower surface. Hagemann (1997) observed the development of only one type of gametangium per tubercle (unisexual tubercles), which suggests that development may depend on culture conditions. Because tubercles of *A. chaerophylla* can develop both antheridia and archegonia, the term "archegoniophore" (Bower, 1923) does not seem appropriate for these structures.

At the green gametophyte-tubercle junction we documented the presence of oversized cells connecting these structures. This kind of cell has not been reported in other ferns. For *Anogramma leptophylla*, Hagemann (1997) mentioned the existence of a prosenchymatic tissue in the contact zone between the gametophyte and tubercle, and suggested the role of these tissues in the transport of substances. Given their position and shape, the oversized

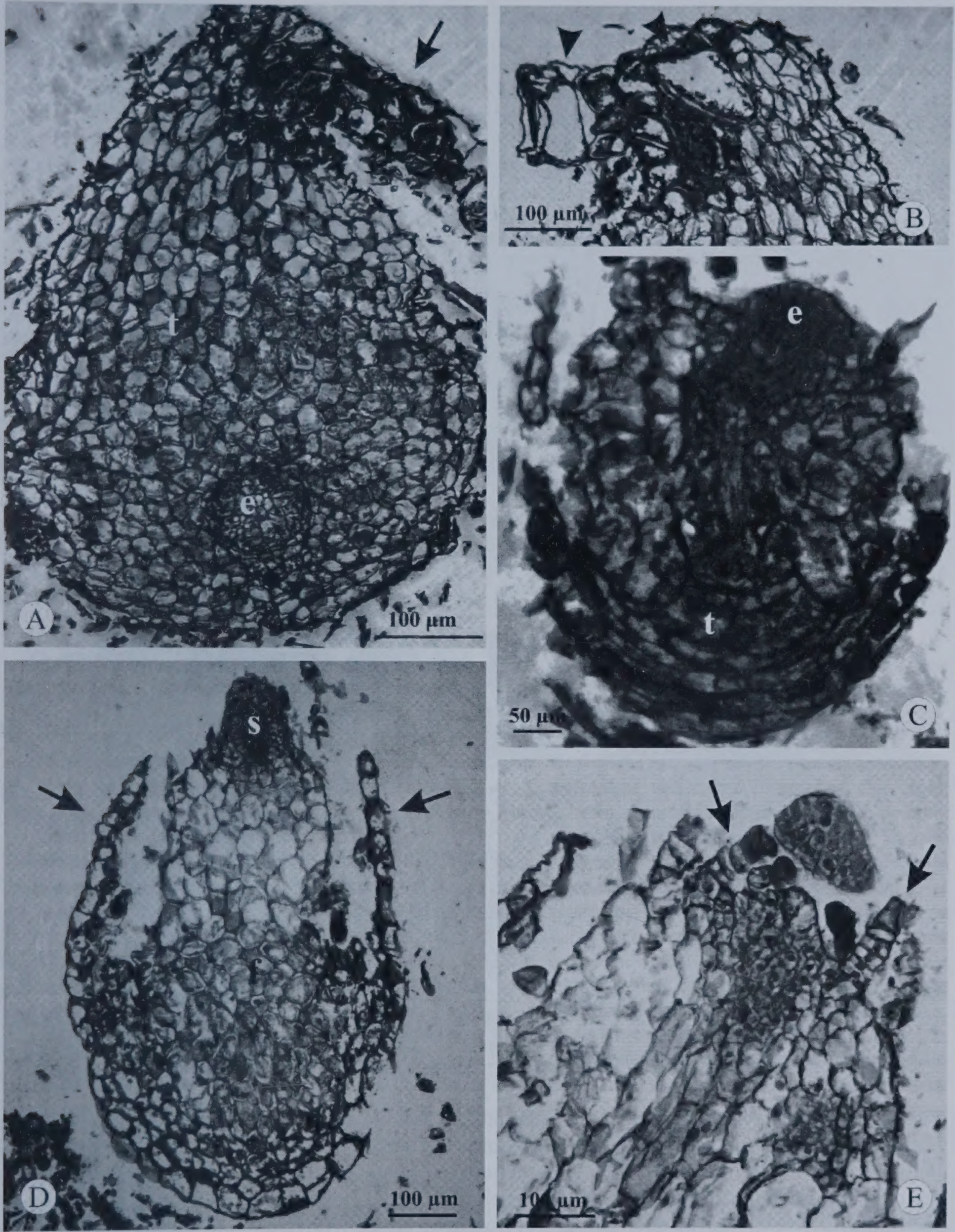

Fig. 3. Embryo development and sporophyte growth in tubercles. A: Section of a tubercle showing the occurrence of only one embryo. B: Detail of oversized cells (arrowheads) at the junction green gametophyte-tubercle. C: An embryo with differentiated shoot apex, suspensor and foot. D: Emergent sporophyte accompanied by calliptra-like outgrowths (arrows). E: Detail of shoot apex with the first developing leaves (arrows) and glandular hairs. Abbreviations: embryo (e), foot (f), sporophyte (s), tubercle (t)

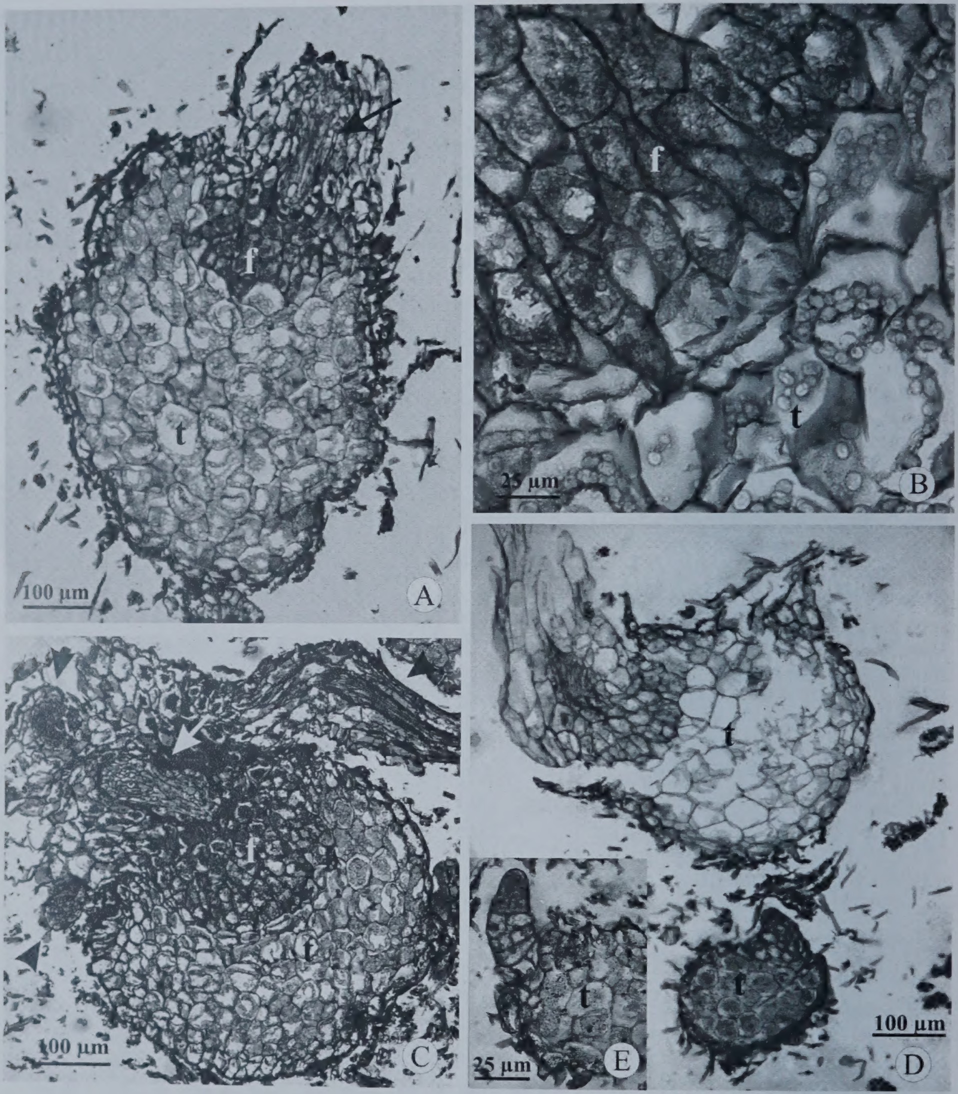

Fig. 4. Morphology of the young sporophyte. A: Tubercle with emerging sporophyte. Vascular tissues are already developed in the short rhizome (arrow). B: Detail of the contact zone (placenta) between the foot and tubercle cells. Note deposition of starch grains in both tissues. C: A more mature sporophyte (arrow) showing three developing leaves (arrowheads). The ones at left appear sectioned. D: Next step of sporophyte development along with complete consumption of the tubercle's food reserves. Note vegetative propagation in a small tubercle below. E: Detail of propagation in tubercle. Note the absence of gametangia. Abbreviations: foot (f), tubercle (t)

cells we observed could correspond to those cells described by Hagemann (1997). Further ultra-structural studies would help to ascertain if they are transfer cells and thus if they take part in the transport of substances between the green gametophyte and the tubercle.

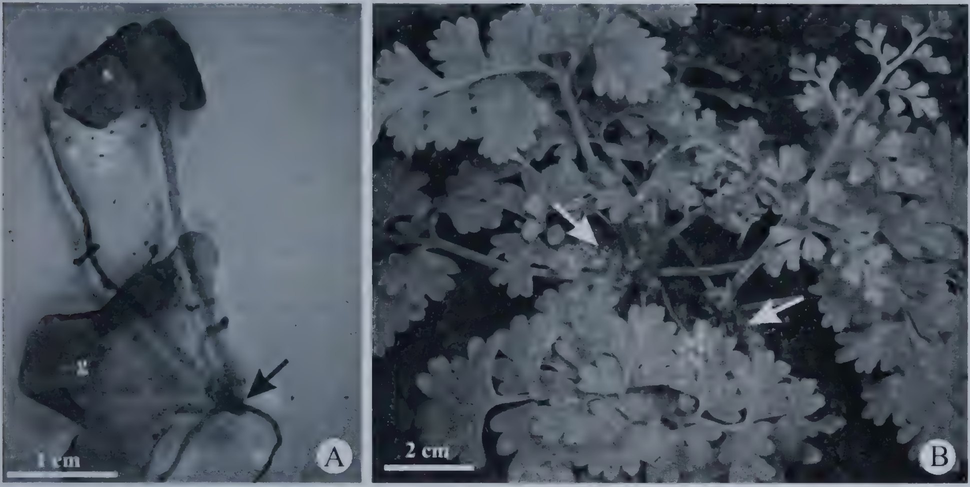

Fig. 5. Macroscopic views of sporophytes grown in laboratory. A: Sporophyte with first developing leaves emerging from the tubercle (arrow). Roots are also observed. B: More mature sporophytes with 4_5 developed leaves. The green prothalli are still visible (arrows). Abbreviations: green gametophyte (g); sporophyte (s)

Successful sporophytes developed exclusively in association with the tubercles, with only one sporophyte forming on each tubercle. Sporophyte growth was accompanied by the gradual consumption of the starch grains accumulated in the tubercle cells, in agreement with the findings of Pangua, Ruzafa, and Pajarón (2011) for *Anogramma leptophylla.*

Information on the structure and ultrastructure of the placenta is scarce in ferns. In species analyzed so far, the placentas comprise elongate haustorial sporophyte cells growing into the adjacent gametophyte cells (Duckett and Ligrone, 2003). In the case of *Anogramma chaerophylla* we observed interdigitation between the foot cells and the adjacent gametophyte cells. This coincides in general terms with observations on other leptosporangiate ferns such as *Ceratopteris richardii* Brongn., *Gleichenia* Sm. and *Pteridium* Gled. ex Scop. (Duckett and Ligrone, 2003; Johnson and Renzaglia, 2009). However, in the case of *A. chaerophylla* the gamethophytic tissue corresponds to the tubercle cells, whereas in the other ferns contact is established with the green gametophyte cells. Further ultra-structural studies in *Anogramma* would contribute to extending the knowledge on fern placentas.

The shoot of the young sporophyte protruded quickly from the tubercle and the first leaf developed early as a photosynthetic organ. Following Gifford and Foster (1989), this type of sporophyte growth enables early synthesis of its own nourishment. The rapid development of the *Anogramma chaerophylla* sporophyte would be related to its annual condition, allowing it to release the spores shortly before dying.

As another adaptive strategy, the foot cells came into contact with the tubercle storage cells and extended their haustorial function until the development of the fourth leaf. In ferns the foot serves as haustorial organ that attaches the embryo to the nutritive tissue of the gametophyte (Gifford and Foster, 1989). The presence of a fairly large foot is usual in embryos that depend nutritionally on the gametophyte for a relatively long time (Johnson and Renzaglia, 2009). Our findings in this species highlight the deep sporophyte-gametophyte nutritional dependency—via the tubercle in this case—when compared to other ferns. The fact that embryos originating in the green gametophyte portion, which lack the reserves of the tubercle, did not prosper supports this hypothesis.

We also documented vegetative propagation of *Anogramma chaerophylla* via smaller tubercules. It is known that tubercles can regenerate the green thalloid part of the gametophyte (Baroustis, 1976; Mehra and Sandhu, 1976; Hagemann, 1997). The main function of the tubercles, as proposed Goebel (1889), would be to keep populations in the gametophytic phase until conditions permitting sporophyte development occur. Our observations of very few sporophytes developed in the field would agree in principle with this hypothesis.

Future directions of study are to document environmental conditions in the study area (i.e., water availability, irradiance, temperature, and soil pH), to gather valuable information concerning what natural populations require to survive. Also, as studies on development of sexuality in gametophytes are conducted mostly in cultural conditions (Ranker and Geiger, 2008), investigations in natural gametophyte populations would contribute to a better understanding the modes of reproduction in *Anogramma.*

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Presence of Juvenile Club Moss (Lycopodiaceae) Sporophytes and Gametophytes in Relation to Vegetation Cover in Dry Pine Forests

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ABSTRACT.—Habitat disturbance is thought to be an important determinant of juvenile club moss population occurrence, yet the few exploratory studies on juvenile club moss populations failed to address the relationship of subterranean gametophytes with incipient sporophytes and aboveground vegetation. Understanding how vegetation influences the development of the juvenile club moss population is crucial for establishing protection for these archaic plants. We used non-metric multidimensional scaling to observe vegetation differences in nine sites with juvenile club moss populations. The vegetation change over a four-year period in a dry pine forest site with a juvenile club moss (Lycopodiaceae) population and nine additional sites used for gametophyte search were analyzed. Sites with emerging juvenile club moss populations in dry pine forests included the following characteristics: 1) vegetation composition stability; 2) no tree canopy shading; and 3) the presence of *Deschampsia flexuosa* (L.) Trin. Other probable factors that might affect juvenile club moss population occurrence in dry pine forests are discussed.

KEY WORDS.—*Lycopodium*, *Diphasiastrum*, gametophytes, juvenile sporophytes, Lithuania

The monophyletic family Lycopodiaceae *sensu lato* comprises approximately 400 living species (Øllgaard and Windisch, 2014). In various temperate forests, homosporous herbaceous lycophytes have an opportunistic, guerrilla-type growth strategy (Harper, 1985) and their evergreen perennial vascular sporophytes, often referred to as "club mosses," form large clones. Despite considerable general interest in these archaic plants, the development and function of juvenile club moss populations has not been studied. The life cycle of club mosses involves an alternation between two generations: asexual sporophyte and sexual gametophyte. However, few studies addressed the development of club moss gametophytes and their habitat specificity. Both generations are critical for species survival and, from a genetic perspective, sporophytes could be considered the less important generation (Haufler, 2002). Juvenile club moss populations are thought to be spatially isolated from old clones, have different abiotic and biotic habitat requirements, and emerge in sites with limited habitat disturbance (Bruchmann, 1898; Degener, 1924;

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* corresponding author

Rimgailė-Voicik *et al.*, 2015). The presence of fire adaptations is also expected (Eames, 1942; Naujalis, 1995; Oinonen, 1968).

Recently there has been growing interest in gametophyte anatomy and development (Renzaglia and Whittier, 2013; Whittier, 2005), but few primary investigations have been performed on juvenile sporophytes and gametophytes in nature. Subterranean club moss gametophytes form in the humus horizon, at a depth of one to nine cm (Bruchmann, 1898; Degener, 1924; Eames, 1942; Naujalis, 1995; Rimgailė-Voicik *et al.*, 2015). Bisexual club moss gametophytes develop slowly and reach maturity after five to ten years (Bruchmann, 1910; Horn *et al.*, 2013). Mature gametophytes of *Lycopodium* L. species are typically shaped like an irregular bowl, and less commonly is carrot-beetroot shaped in the genus *Diphasiastrum* Holub (Bruchmann, 1898; Horn *et al.*, 2013; Rimgailė-Voicik and Naujalis, 2015; Thomas, 1975). Juvenile sporophytes have a prolonged period of matrotrophy and are dependent on the gametophyte for several years (Renzaglia and Whittier, 2013). Achlorophyllous gametophytes are associated with fungal endophytes (Read *et al.*, 2000), but the true nature of fungi-gametophyte relationships remains unclear. For example, recent research on *Diphasiastrum alpinum* (L.) Rothm. (Horn *et al.*, 2013) supports a hypothesis that gametophytes are mycotrophic holoparasites.

This paper characterizes the vegetation change and composition of a juvenile club moss population over four years. We also analyze relationships among different forest sites containing gametophytes and juvenile sporophytes, and discuss factors that might influence development of juvenile club moss populations. The aims of this study were to: 1) analyze the structural composition and vegetation change over four years in a dry pine forest habitat with a juvenile club moss population; 2) test whether juvenile sporophytes found in communities represent different or similar stages of succession; 3) investigate if juvenile sporophytes inhabit different microniches; and 4) address multivariate differences among sites, vegetation cover, number of juvenile sporophytes, and presence of subterranean gametophytes.

MATERIALS AND METHODS

Study site.—Research was conducted in a *Pinus sylvestris* L. dominated forest of the Varėna District in southern Lithuania. Forests occupy about 68.9% of the Varėna District, and pure pine forests with *P. sylvestris* or mixed pine forests with *Betula* sp., *Quercus robur* L., and *Picea abies* (L.) H. Karst are present. The forests are a type of dry pine as attributed to ass. *Peucedano-Pinetum* W.Mat. (1962) 1973 (Matuszkiewicz, 2001). Mean absolute temperature in January is –4.2 °C and +17 °C in July. Mean annual precipitation is 670 mm; snow cover lasts for 90 days. Hilly moraine relief soils are mostly podzolic light (sandy or sandy loam; Anonymous, 2015). Five species of club mosses were present in the studied forests: *Lycopodium annotinum* L., *L. clavatum* L., *Diphasiastrum complanatum* (L.) Holub, *Diphasiastrum tristachyum* (Pursh) Holub, and *Diphasiastrum* × *zeilleri* (Rouy) Holub. Field research was conducted every year from 2012 to 2015 in

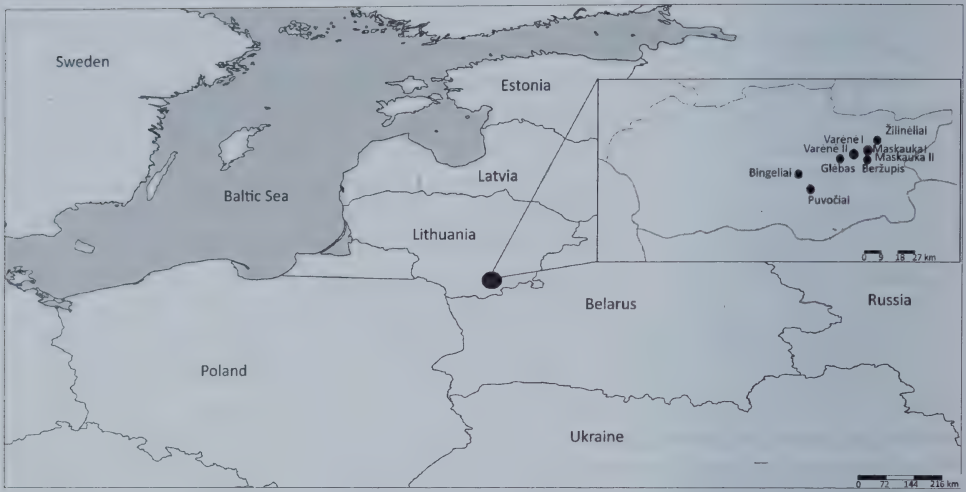

Fig. 1. Map of the study area in the Varėna District, Lithuania. The samples for a gametophyte search were collected from nine different sites (Maskauka I, Maskauka II, Varėnė I, Varėnė II, Puvočiai, Bingeliai, Beržupis, Žilinėliai, Glėbas) and Maskauka site was selected for repeated assessment of vegetation cover and juvenile sporophytes. Because of a close vicinity, Maskauka for repeated assessment and Maskauka I and Maskauka II are represented as one dot as well as Varėnė I and Varėnė II.

June and August and began with an initial search for juvenile club moss populations in dry pine forests near Senoji Varėna village.

The Maskauka site (N54.28202, E024.59892 WGS) had the largest cover of juvenile sporophytes and was selected for repeated assessment of vegetation cover and juvenile sporophytes. Nine sites were selected to search for gametophytes (Fig. 1): Maskauka I (N54.28547, E024.60547), Maskauka II (N54.28029, E024.59781), Varėnė I (N54.26412, E024.53109), Varėnė II (N54.26451, E024.53276), Puvočiai (N54.11251, E024.31869), Bingeliai (N54.17616, E024.26510), Beržupis (N54.23603, E024.59468), Žilinėliai (N54.32018, E024.64004), Glėbas (N54.24304, E024.46539).

Study subjects.—We investigated these components of juvenile club moss populations: subterranean gametophytes, sporophyte sporelings (still with their developing gametophytes), and juvenile sporophytes (already independent of their gametophyte).

The sampling design.—At the permanent Maskauka site, vegetation coverage and the location of juvenile sporophytes were recorded at one-year intervals, from 2012 to 2015, in August. For visual cover evaluation, a plot of 100 m^2 was marked and divided into 1 m^2 subplots with wooden dowels (Fig. 2). Vegetation cover was evaluated using percentage scale in 100 subplots of 1 m^2 in size. In 2013 and 2014, the development of juvenile sporophytes was evaluated by counting the number of shoots seen above the moss layer in 100 subplots. In August of 2015, tree canopy projection was performed and the

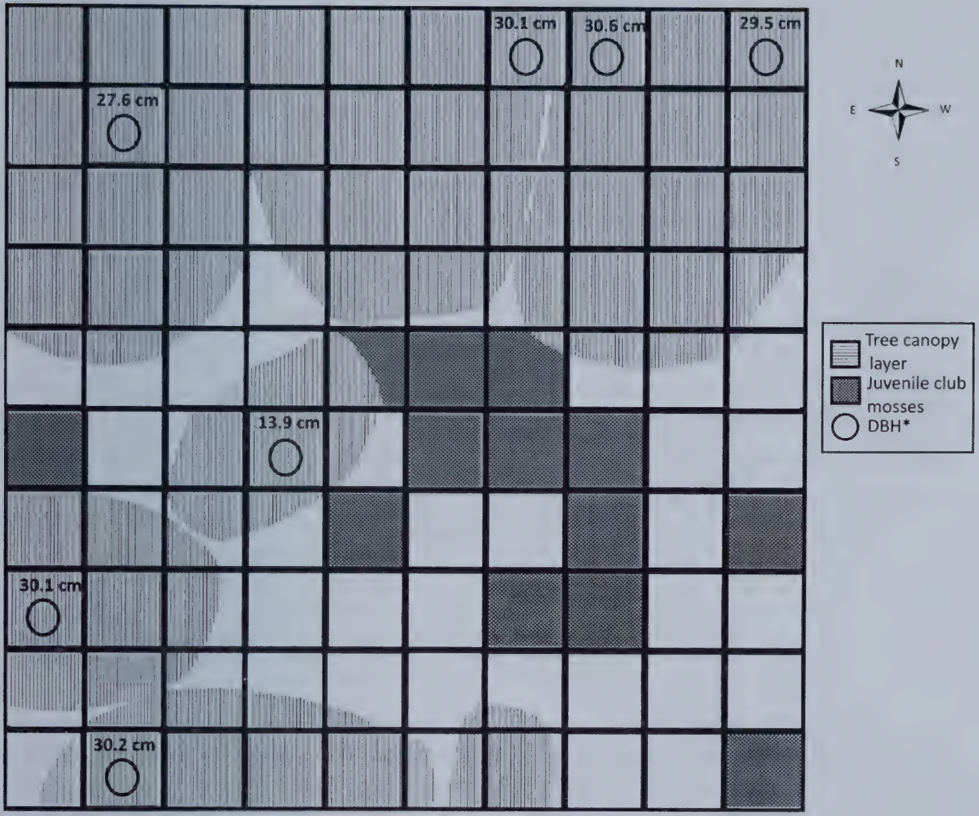

FIG. 2. Scheme of the permanent Maskauka site of 10 × 10 m^2 with a juvenile club moss population in 2015. The research site of 100 m^2 was marked and divided into 1 m^2 subplots with wooden dowels. Tree canopy projection was performed. During annual fieldwork, the location of sporophyte sporelings and juvenile sporophytes within 1 m^2 subplots and their developmental stage were determined. In 2015 soil samples within subsamples with juvenile club moss sporelings were collected. * tree diameter at breast height.

diameter at breast height (DBH) was measured. *Pinus sylvestris* trunks fell into seven research subplots out of 100; these subplots were omitted from analysis.

Juvenile sporophyte analysis.—At the end of August 2015, all juvenile sporophytes at the Maskauka site were excavated with 10 × 10 × 10 cm soil samples. Soil samples were gradually disassembled with tweezers, to search for gametophytes and extract juvenile sporophytes. The size of the identified juvenile sporophytes was described and their age, according to number of annual constrictions (Primack, 1973), was determined (Fig. 3). During this research, we also determined the number of shoots and their growth pattern (orthotropic or plagiotropic).

Assessment of subterranean gametophyte populations.—In 2012, a soil sample of 50 × 50 × 10 cm with intact forest floor was collected near the permanent Maskauka site. In the laboratory, the soil sample was gradually

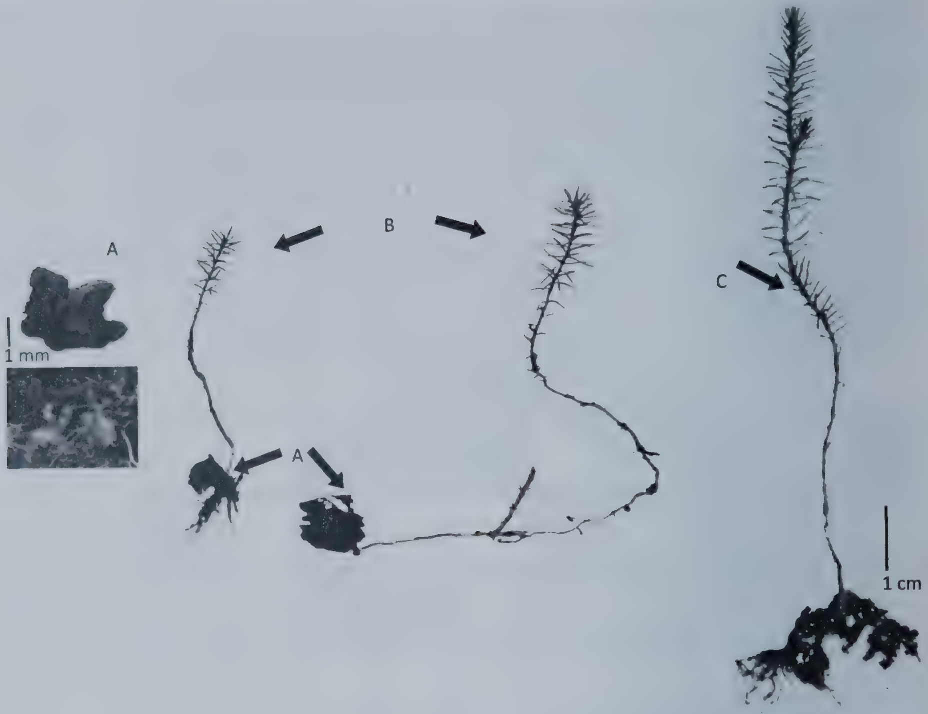

Fig. 3. Stages of club moss sporophyte development. A – subterranean gametophytes; B – sporophyte sporelings; C – annual constriction of juvenile sporophytes.

disassembled with tweezers to search for gametophytes using a modified method for quantifying soil invertebrates (Ghilarov and Striganova, 1987). The number of juvenile sporophytes and gametophytes was recorded, and the developmental stage of each individual was evaluated. During 2013 and 2014, 24 soil samples (50 × 50 × 10 cm; three samples in each of the nine sites) in dry pine forest localities were collected and analyzed. All sampling sites were located in Varėna District, 1-20 km from the Maskauka site. Samples were taken in a transect through the locality with juvenile sporophytes and were analyzed using the previously described method. Vegetation cover was evaluated visually using percentage scale.

Data analysis.—All statistical analyses were performed in R using statistics Package BiodiversityR vegan (Oksanen *et al.*, 2016). Nonmetric multidimensional scaling (NMDS) was performed to test for differences in vegetation among samples collected for gametophyte search and to explore probable patterns of subterranean gametophyte emergence in relation to vegetation composition. Distances were calculated using the vegdist function for Bray-Curtis.

Permutational MANOVA was applied to test for significant differences among forest sites in the number of juvenile sporophytes and gametophytes found. The Permutational MANOVA generated Bray-Curtis and Hellinger dissimilarity measures. Results were calculated with the function adonis (Oksanen *et al.*, 2016) in the package vegan. For the Maskauka site, a permutational MANOVA was used to test if there were no differences in subplots with and without tree canopies.

Shannon and Simpson indexes were subjected to repeated measures ANOVA (RM-ANOVA) with years as within-subject factors. Significance level was adjusted using the Greenhouse-Geisser correction. Significant main effects and interaction effects were followed up with post hoc pairwise comparisons adjusted using a Bonferroni correction. The calculation was performed with the function ezANOVA in the package ez (Lawrence, 2011).

Species richness accumulation curves were generated, and species rank-abundance values were calculated. For the evaluation and comparison of diversity at our study sites, Renyi diversity profiles among nine field sites, and among the vegetation composition data over four years in the permanent Maskauka site (2012-2015) were generated. Coverage data per species was transformed from percentage to relative coverage.

The Renyi diversity profile was suggested (Renyi, 1961) in the form:

$$H_\alpha = \frac{1}{1-\alpha}\ln(\sum_{i=1}^{S} p_i^\alpha)$$

where p_i is the relative abundance of *i-th* species and S is a total number of species in the sample. An equation is interpreted for the range of scale parameter α: $\alpha \geq 0$, $\alpha \neq 0$. This method considers several diversity indices and binds them into a coherent framework. Renyi's entropy is related to a wide range of diversity indices by a power law—the Hill number (Na = eHa) and many diversity indices are special cases of the Hill number (Hill, 1973). The Renyi index estimates total richness for $\alpha = 0$, Shannon-Wiener index for $\alpha = 1$, the inverse Simpson's-Yule index for $\alpha = 2$ and 1/Berger-Parker or dominance index for $\alpha = \text{Inf}$ (Oksanen *et al.*, 2016). Extended use of this method is suitable for conservation biology and interpretation of diversity by placing various weights on different abundance categories (Lövei *et al.*, 2013).

Correlation matrices were generated to test for significant correlations among different species coverage during the four-year period in the Maskauka site and among the nine forest sites where gametophyte presence vs. absence were determined. Pearson correlation coefficient was calculated between the number of gametophytes and the number of juvenile sporophytes detected.

Chemical analysis.—A humus horizon soil sample for chemical analysis was created by combining three subsamples taken in the Maskauka site in August of 2015. Soil samples for juvenile sporophyte analysis and chemical analysis in permanent research subplots were collected only in 2015 so that the sampling would not affect the vegetation. Soil analyses were performed at the National Public Health Surveillance Laboratory, located in Vilnius, Lithuania. Soil pH was determined in an aqueous suspension (ISO 15933), total amount

TABLE 1. Characteristics of the vegetation in a 100 m^2 research plot with juvenile Lycopodiaceae sporophytes and gametophytes in the Varéna District of Lithuania (54.28202, 24.59892 WGS).

Characteristics	2012	2013	2014	2015
Number of quadrats* with juvenile club moss individuals in 2012	12	11	13	13
Average ± SD of herb-subshrub layer cover (%),	34 ± 23	35 ± 23	34 ± 23	36.3 ± 15
Average ± SD cover of moss-lichen layer (%)	95 ± 14	95 ± 13	95 ± 14	98 ± 9
Absolute abundance of the most abundant species:				
Pleurozium schreberi	52.1	51.7	54.4	52.9
Deschampsia flexuosa	20.6	21.3	20.3	20.4
Dicranum polysetum	17.5	17.5	15.1	14.0
Vaccinium myrtillus	1.1	1.1	1.4	2.7
Vaccinium vitis-idaea	0.7	0.7	0.8	1.5
Number of vascular plant species	7	8	10	13
Number of moss-lichen species	6	4	6	5
Total estimated species richness	13	12	16	18

Total number of quadrats was 100. SD – standard deviation

of nitrogen was determined according to the Kjeldahl method (ISO 11261) and spectrometric determination was used to obtain total phosphorus (ISO 11263).

RESULTS

Plant community analysis in the Maskauka permanent research plot.—The flora in the permanent Maskauka site was not particularly species-rich, and over four years, species richness varied slightly (Table 1). The species accumulation curve for the Maskauka site reached an asymptote in 2012 after less than 50 subplots out of 100 were sampled. In all other years, an asymptote was never reached. The Renyi diversity profile indicated that the evaluated years cannot be ranked, the highest diversity according to species richness was in 2015, but when common species had more weight (Simpson's-Yule index for $\alpha = 2$), all years were similar.

The average coverage of grass-subshrubs in subplots with juvenile sporophytes was 46%. *Deschampsia flexuosa* was identified in 96 subplots. *Deschampsia flexuosa* appeared together with *Lycopodium annotinum* in all subplots (average cover of 35%). The correlation matrix showed a weak, but significantly positive correlation between *D. flexuosa* and *L. annotinum* cover ($r_{xy} = 0.22$; $p < 0.05$) using average cover over four years in the calculation. No significant correlation was detected between *L. annotinum* and any other species.

Statistical analysis revealed that subplots not shaded by the tree canopy were more stable in regards to species diversity and abundance, and did not change significantly during the research period. Juvenile lycopods were identified in subplots that were not shaded. The subplots with and without tree canopy shading significantly differed from 2012 to 2014 (permutational MANOVA on Bray-Curtis distances; $R2 = 0.05$; $p < 0.01$), but not in 2015. The Shannon index among subplots in different years differed significantly (repeated measures ANOVA with a Greenhouse-Geisser correction; F = 19.1649; $p < 0.0001$). To detect where the difference emerged, post hoc tests using the Bonferroni correction was applied and results revealed that only 2015 significantly differed from all other years ($p < 0.01$). Further analysis showed that the Shannon index significantly differed between time points (repeated measures ANOVA; F = 18.08; $p < 0.0001$) and when subplots with only tree canopy cover were included in the analysis. Simpsons index also differed significantly between time points (repeated measures ANOVA; F = 18.17; $p < 0.0001$) and post hoc tests using the Bonferroni correction revealed that only 2015 significantly differed from all other years ($p < 0.01$). Also, Simpson's index differed significantly between years (F = 18.08; $p < 0.0001$) when only subplots with tree canopy cover were included.

In the Maskauka permanent research plot, the tree canopy layer was around 40%, and the shrub layer was absent. In subplots with juvenile club moss sporophytes, no pine trunks were present and the canopy did not shade club moss sporophytes. For most subplots, the grass-subshrub layer covered more than 50% and remained unaffected. Club mosses were determined to cover 10% of the study site; the average cover increased from 2% in 2012 to 3% in 2015.

Every year in the Maskauka subplots seedlings of *Frangula alnus* Mill., *Betula pendula* Roth, *B. pubescens* Ehrh., *P. sylvestris*, *Juniperus communis* L., *Quercus robur* were observed. One *Q. robur* seedling remained throughout the study, but other seedlings perished. Only *P. sylvestris* seedlings occurred in subplots with juvenile club mosses, but they did not overwinter.

The total cover of mosses and lichens was close to 100%. Only *Pleurozium schreberi* (Brid.) Mitt. occurred in all subplots together with juvenile sporophytes, average cover of *P. schreberi* was 88%. *Dicranum polysetum* Sw. had an average coverage of 10% in nine out of 14 subplots with juvenile club mosses present. In one subplot, juvenile sporophytes occurred together with *Dicranum scoparium* Hedw. (coverage: 10%). Even though statistical analysis showed that subplots with juvenile club mosses had stable vegetation, in these subplots new moss species were detected that did not overwinter or were displaced by other species. In 2012 in one subplot, *Hylocomium splendens* (Hedw.) Schimp. (coverage 5%) occurred, and in 2014 in one subplot, *Polytrichum piliferum* Hedw. occurred.

Juvenile club moss sporophytes in the Maskauka permanent research plot.— Throughout our research, juvenile club moss sporophytes were detected in 14 out of the 100 assessed subplots (Table 2; Fig. 2). Juvenile *L. annotinum* dominated the subplots; *L. clavatum* growing with *L. annotinum* was only present in one subplot. *Diphasiastrum* species did not occur. In 2012, we observed 35 shoots above the moss layer in twelve subplots. Three of the 35 observed shoots had both orthotropic and plagiotropic growth in 2012, in other years no drastic changes were observed.

In 2015, 20 soil samples were analyzed and 23 juvenile sporophytes were found: 22 *L. annotinum* and one *L. clavatum*. Additionally, three juvenile *L. annotinum* sporophytes were found dead and covered with mosses. In the

TABLE 2. Data on juvenile sporophytes collected from the permanent research subplot in 2015.

Characteristics	Average and SD	Max	Min
Moss layer thickness, cm	4.65 ± 1.87	9	2
Humus layer, cm	2.36 ± 0.97	4	0.8
Number of juvenile sporophytes per sample*	1.21 ± 0.71	4	1
Number of annual constrictions	3.68 ± 1.55	8	2
Number of roots	2.52 ± 1.16	6	1
Number of branches	4.04 ± 3.5	13	1
Distance between branches	2.43 ± 1.77	17	0.8
Length of branches	3.27 ± 2.97	12.3	0.2
Length of juvenile sporophytes	16.39 ± 7.87	39.9	7.2

* A total of 23 juvenile sporophytes of different developmental stages were found.

humus horizon of one sample, two irregular bowl shape gametophytes (Type I, *Lycopodium clavatum* type, according to Bruchmann, 1898: *Lycopodium* sp.; 1.5 × 1.8 cm and 0.4 × 1.2 cm) were found at a depth of 0.2 cm. Nine out of 23 juvenile sporophytes had orthotropic growth, and the remaining 14 had both orthotropic and plagiotropic growth. The length, branching pattern and other growth traits were uneven (Table 2). According to shoot branching, the population of juvenile sporophytes was attributed to three groups that resembled different developmental stages: 1) juvenile sporophytes with orthotropic branchless shoots; 2) juvenile sporophytes with orthotropic shoots and short branches; and 3) juvenile sporophytes with plagiotropic and orthotropic growth.

Assessment of subterranean gametophyte populations.—In the test soil sample, collected in Maskauka, 113 subterranean gametophytes of different developmental stages and 17 juvenile sporophytes were found. Eighty gametophytes were Type I and 33 gametophytes were Type II (*Diphasiastrum complanatum* type according to Bruchmann, 1898). The largest portion (54%) of juvenile sporophytes had intact gametophytes, but were photosynthesizing.

The number of gametophytes found in nine sites assessed varied from two to 133. In 11 out of 24 samples, no gametophytes were found. The Pearson correlation coefficient showed moderate positive association between the number of gametophytes and sporophytes ($r_{xy} = 0.57$; $p < 0.05$). We used species cover data to test for correlation among different species. A weak positive significant correlation was found between *D. flexuosa* and *L. annotinum* ($r_{xy} = 0.39$; $p < 0.05$). *Deschampsia flexuosa* and *Vaccinium myrtillus* L. ($r_{xy} = -0.41$; $p < 0.05$), *L. annotinum* and *V. myrtillus* ($r_{xy} = -0.37$; $p < 0.05$) and *D. flexuosa* and *D. polysetum* ($r_{xy} = -0.45$; $p < 0.05$) had moderately negative Pearson correlations. NMDS (Fig. 4) showed that the samples with a larger number of gametophytes were more similar and that increasing number of juvenile club moss sporophytes is related to a larger subterranean gametophyte population in a sample.

No gametophytes were found in any Beržupis and Puvočiai samples although juvenile sporophytes were present. All Beržupis samples, two

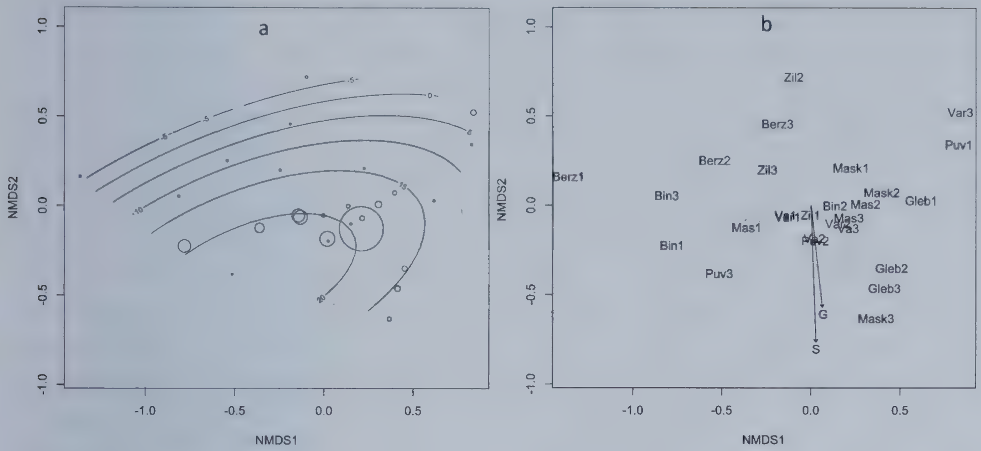

FIG. 4. Multivariate structure of the dry pine forest communities with juvenile club moss populations based on Bray-Curtis distances: a) samples plotted with nonmetric multidimentional scaling (NMDS); b) contour lines representing the average number of gametophytes found, which was fit after the ordination. S and G vectors represent positive relationship between the increasing number of juvenile sporophytes and gametophytes in a sample. Bin1, Bin2, Bin3 – Bingeliai samples; Gleb1, Gleb2, Gleb3 – Glėbas samples; Berz1, Berz2, Berz3 – Beržupis samples; Zil1, Zil2, Zil3 – Žilinėliai samples; Var1, Var2, Var3 – Varėnė II samples;Va1, Va2, Va3 – Varėnė I samples; Mas1, Mas2, Mas3 – Maskauka I samples; Mask1, Mask2, Mask3 – Maskauka II samples; Puv1, Puv2, Puv3 – Puvočiai samples.

Bingeliai samples, and two Žilinėliai samples were more scattered in the NMDS plot, with a Kendall's stress value of 7% (Fig. 4). Vegetation data of other samples tended to cluster. No gametophytes were found in one out of three samples in the Maskauka II, Varėnė II, Bingeliai, Žilinėliai, and Glėbas sites. Puvočiai samples were scattered in all plots and seemed to be very different from each other. According to the Renyi diversity profiles (Fig. 5), the nine field sites cannot be ranked, as curves cross each other or intersect at least once. The Žilinėliai site had the highest species richness and lowest coverage of the most abundant species, the same results were seen in the NMDS plot.

In total, 381 gametophytes were found in 24 samples. The proportion of Type I and Type II gametophytes was 89% (340) *Lycopodium* sp. and 11% (41) *Diphasiastrum* sp. gametophytes. Two hundred and seven juvenile sporophytes without gametophytes were found: seven were *D. complanatum* (determined by Karsten Horn), 195 were *L. annotinum* and five were *L. clavatum*. No significant relationships among the number of gametophytes, number of juvenile sporophytes and vegetation diversity were found.

Soil chemical analysis.—The chemical analysis of the top 0–10 cm of soil in the permanent research site showed that soil was acidic (pH 5), total nitrogen was 1.3 ± 0.11 g/kg, and the total phosphorus was 10 ± 1 mg/kg.

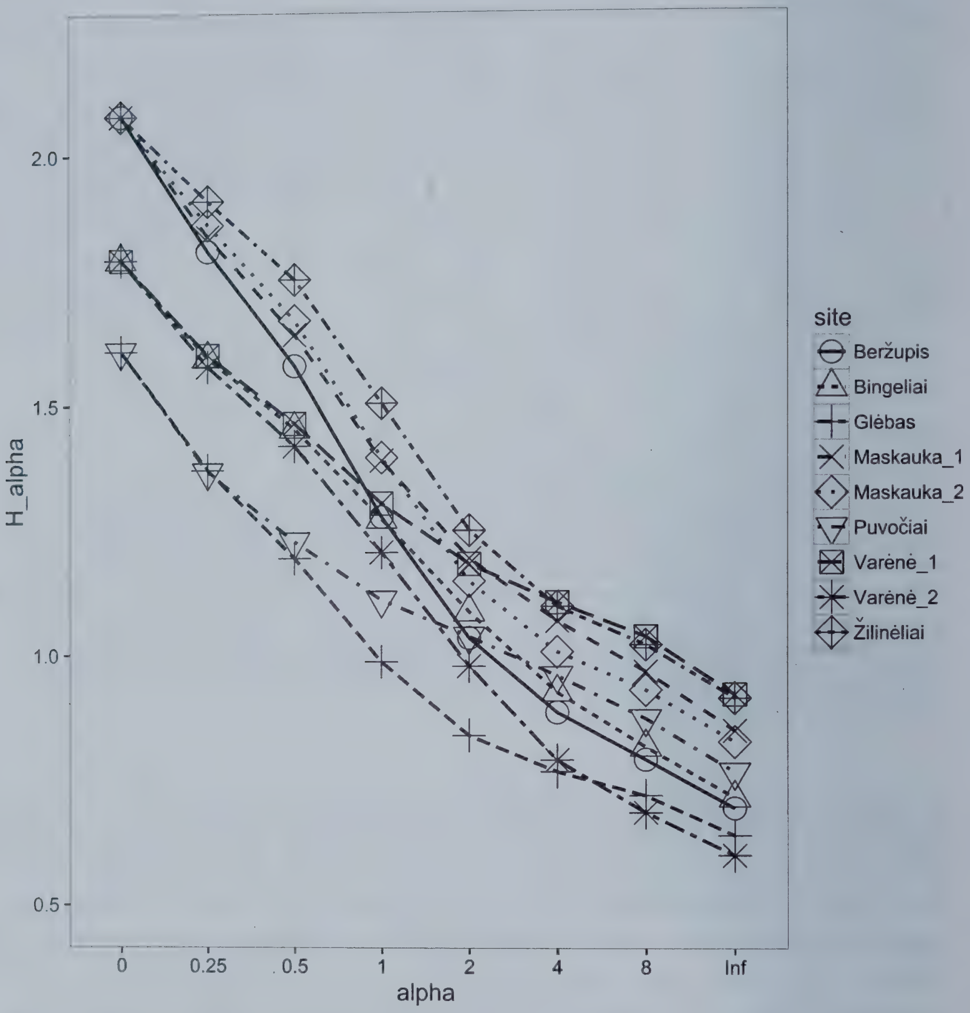

FIG. 5. Renyi diversity profiles of nine sites with juvenile club moss populations. The scale parameter (α) gives the order of Renyi diversity; $\alpha = 0$ is the logarithm of species richness, $\alpha = 1$ is the Shannon diversity index, $\alpha = 2$ is the logarithm of the reciprocal Simpson's diversity index, $\alpha =$ Inf refers to the proportion of the most abundant species. The x and y-axis show, respectively, the alpha value of the Renyi's formula and their associated Renyi diversity profile values.

DISCUSSION

The dry pine forests we studied in the Varėna District of Lithuania were not species rich and had no shrub layer. In sites dominated by the grass *Deschampsia flexuosa*, no adult club moss clones were present in close proximity. The Renyi diversity index (Lövei *et al.*, 2013) indicated that forest sites were slightly stressed and disturbed. We propose that all of the above listed characteristics made the researched forests suitable to support juvenile club moss populations.

Development of the juvenile club moss populations depend on other nearby species in the community. We found a weak significantly positive correlation between *D. flexuosa* and *L. annotinum* cover in the Maskauka site, but no significant correlations with other species were detected. Ódor (1996) argued that *L. clavatum* and *D. complanatum* can easily coexist with *V. myrtillus*. Our study showed weak negative correlation of *L. annotinum* and *V. myrtillus*, but slight positive correlation of *L. annotinum* and *D. flexuosa*. In dry pine forests of the Varėna District, *D. flexuosa* predominates over the subshrubs *V. myrtillus* and *Vaccinium vitis-idaea* L. However, 40 years ago *D. flexuosa* was considered a rare species in Lithuania (Natkevičaitė-Ivanauskienė, 1963). Ruotsalainen *et al.* (2007) showed that *D. flexuosa* can become dominant in disturbed forests. Species composition in the Maskauka site did not change over our four-year study, but reappearing tree seedlings show the presence of open microniches.

Apparently, juvenile sporophytes inhabit different ecological microniches in the same community. Øllgaard (1985) noted that club moss gametophytes thrive in situations where their sporophytes perish, because the subterranean habitat is much more uniform compared with surface habitats. The emergence of microniches is mainly caused by human and animal activities. Previously, subterranean gametophytes and juvenile sporophytes were found on and near forest roads, tracks, near lines separating forest blocks, and skiing tracks (Bruchmann, 1898; Degener, 1924; Horn *et al.,* 2013; Muller *et al.,* 2003; Stokey and Starr, 1924). In Lithuania, juvenile club moss populations also occur in previously disturbed sites (Naujalis, 1995; Rimgailė-Voicik *et al.,* 2015). According to Stokey and Starr (1924), all localities with juvenile club moss populations can be described as "poor collecting grounds" and fall into three main types: 1) grove of mixed hardwoods on a slope or near the slope, above a body of water; 2) relatively dry depressions in a grove of mixed hardwoods; or 3) in a hemlock grove. Eames (1942) noted that a prosperous juvenile club moss population was discovered in a forest with trees and schrubs of 5–25 years old, grown after a forest fire following timber cutting. Juvenile club moss populations were spatially isolated from old clones (Fankhauser, 1873; Bruchmann, 1898; Spessard, 1922). Our results support these findings.

Presumably, microniches form because of the specific soil fungi present. The association of subterranean or partly subterranean Lycopodiaceae gametophytes with fungi has been proposed since the 1800s and might be responsible for normal gametophyte development (Bruchmann, 1898; Schmid and Oberwinkler, 1993; Treub, 1884), but knowledge on these associations is remarkably limited. The formation of AM (arbuscular mycorrhizal) mycelial networks between subterranean club moss gametophytes and *D. flexuosa* remains unclear and further research is needed. Based on Internal Transcribed Spacer (ITS) and large subunit ribosomal RNA (LSU) sequence data, Horn *et al.* (2013) hypothesized that *D. alpinum* mycoheterotrophic gametophytes participate in the mycorrhizal symbiosis and obtain nutrients from *Calluna vulgaris* L. via a fungal connection. Experimental evidence is needed to show whether AM mycelial networks could connect gametophyte and sporophyte generations and allow autotrophic or other species to supply carbohydrates to club moss gametophytes (Leake *et al.*, 2008).

More obvious microniches may appear because of the activity of ants. Although the impact of ants on juvenile club moss populations remains unclear, some evidence suggests that ants have an impact on gametophyte development. In soil samples where ant nests were large, few or no gametophytes were present. We observed ants moving grains of sand, sometimes forming up to a 10 cm layer of sand on top of the humus. In soil samples taken during our gametophyte search, we identified the most common ant as *Lasius flavus* (Fabricius; *Formicidae*), which is common in temperate regions of Europe and live mostly underground (Dostal, 2007). According to Bruchmann (1898), club moss spores easily stick to the various surfaces. Arthropods can move a huge number of spores into deeper layers of the soil as well. In a soil sample of 1 m^2 in size in a dry pine forest of the Varėna District, more than 110 arthropods belonging to 54 species have been identified (Eitminavičiūtė, 2001).

In the Maskauka permanent research plot only three juvenile sporophytes perished. The mortality rate in the juvenile club moss population was low. No comprehensive data on death rates of gametophytes and juvenile sporophytes has been previously published and little is known about the mortality rate of juvenile sporophytes. According to Stokey and Starr (1924), juvenile sporophytes would be more likely to occur after 10–12 years without a drought. During observations over seven years (1985–1991) in dry pine forests, we discovered that more than 50% of *L. annotinum* juvenile sporophytes died, and the annual growth rate was about 1–3 cm (Naujalis, 1995). As terrestrial gametophytes emerge in large groups, mortality rates among them were found to be greater than among club moss gametophytes (Naujalis, 1995). Among ferns with terrestrial bisexual gametophytes, an antheridiogen effect is common and larger gametophytes inhibit the growth of smaller ones, causing high gametophyte mortality rates (Hamilton and Lloyd, 1991). During our four years of research in the Maskauka site, the main reason for juvenile sporophyte death appears to be competition with the moss layer. In temperate forests, the annual growth rate of the moss species *Hylocomium splendens* and *Pleurozium schreberi* was about 2.6 cm and 2.1 cm, respectively (Zechmeister, 1995). If juvenile sporophytes continue to grow faster after a few years, they outgrow mosses, but by then the underground gametophytes would have decomposed.

The different development levels of juvenile club mosses found in our study site supports a hypothesis of asynchronous development within a club moss population and appears to be the main reason for intraspecific competition in a limited area. The overall results indicate that juvenile club moss populations were formed from sporophytes of different ages that emerged from gametophytes and therefore are not likely to be genetically identical. Genetic variability within clones is rarely reported (Major and Ódor 1999; Wittig *et al.*, 2007). If a clone is one individual, after a certain time in juvenile population only one individual would remain alive, but no evidence of it appeared during our four years of research. Cousens *et al.* (1985) suggested that sites for gametophyte development were rare and lead to clustering of spores of many different species in a small territory. This hypothesis is supported by the present study, as we found diverse developmental stages of gametophytes of different species in close proximity to each other. The juvenile club moss population in the Maskauka site may have developed approximately ten to 15 years ago and the development remains continuous, as we found live gametophytes in 2015. According to Grime (1985), club moss sexual propagation is highly inefficient because a long period of development is required to reach maturity. It is believed (Bruchmann, 1910; Horn *et al.,* 2013) that subterranean gametophyte development from spore germination to mature gametophyte bearing antheridia and archegonia might take five or more years.

Forest vegetation humus chemistry is related to understory species and dominant conifers (Bradshaw and Zackrisso, 1990; Mallik, 2003). When comparing chemical soil parameters with long-term research performed in nearby forests of the Varėna District (Armolaitis *et al.,* 2011) and data of Lithuanian forest soil monitoring (Beniušis, 2008), the Maskauka permanent research site can be attributed to pine forests never used as arable soil or fertilized.

Our research on dynamic juvenile club moss populations provided new insight into the development and function of these archaic plant populations in nature. However, many questions remain unanswered. During the four-year field research period, we ascertained that the development of club moss populations is a long-term process, during which the unsynchronized occurrence of juvenile sporophytes from subterranean gametophytes takes place and partial elimination of juvenile sporophytes occurs. Among the gametophytes discovered, the genus *Lycopodium* dominated. There was a moderate positive correlation between the number of gametophytes and juvenile sporophytes present in the soil sample, supporting the hypothesis that the best place to look for gametophytes is where juvenile sporophytes are present. Both in the permanent Maskauka site and in the nine other plots, the cover of *L. annotinum* correlated with cover of *D. flexuosa*, while the cover of *V. myrtillus* negatively correlated with the cover of *D. flexuosa* and *L. annotinum*. Future research should test if *D. flexuosa* shares the same fungal associations as gametophytes and analyze the genetic variation among juvenile sporophytes and adult assemblages.

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Anatomical and Morphological Leaf Traits of Three Evergreen Ferns (*Polystichum setiferum*, *Polypodium interjectum* and *Asplenium scolopendrium*)

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Abstract.—Leaf mass per area (LMA) is one of the key features that correlates with the ecological performance both of seed plants and ferns. For ferns LMA is at the lower end of the range for seed plants and increases with leaf longevity. Data concerning morphological and anatomical traits of ferns and their relationship with LMA are quite limited. The objectives of this study were to (1) break down LMA into anatomical components; and (2) analyze the trade-offs between anatomical and morphological leaf traits, which determine LMA variations for three evergreen ferns. Seven morphological and 16 anatomical leaf traits of *Polystichum setiferum*, *Polypodium interjectum* and *Asplenium scolopendrium* growing outdoors in the Botanical Garden of Rome were analyzed using light microscopy. LMA was not significantly different between the considered species (4.55±0.55, 4.34±0.47, 4.28±0.41 mg cm^{-2} respectively) and it was in the range of other evergreen fern species. The morphological and anatomical structure of all species was significantly different and reflected environmental adaptation of species to their natural habitats. In particular, total lamina thickness was163±16.1 μm (*P. setiferum*), 244±33.7 μm (*P. interjectum*) and 336±32.2 μm (*A. scolopendrium*); leaf tissues density ranged from 127±16.9 mg cm^{-3} (*A. scolopendrium*) to 277±37.1 mg cm^{-3} (*P. setiferum*); intercellular air space varied between 23.2±2.07% (*P. interjectum*) and 41.8±1.61% (*A. scolopendrium*). The overall results highlight that LMA is an integral feature that can be obtained by different anatomical structure.

Key Words.—LMA, leaf tissues density, intercellular air space, stomatal index, leaf life-span.

Ferns are estimated to include 10,578 species worldwide (Pteridophyte Phylogeny Group, 2016) and are most diverse in the tropics especially in wet habitats with moderate air temperature ranging from 1000 to 2500 m a. s. l. (Kessler, 2010). Nevertheless, ferns growing in temperate climates or tropical climates with a dry period show a seasonal leaf phenology (Sharpe and Mehltreter, 2010). According to Sato (1982) ferns from northern temperate regions are classified as evergreen, winter-green, semi-evergreen, and summer-green, in relation with the presence of leaves in different periods during the year. Leaf life-span is linked with morphological and anatomical traits. Among morphological traits, leaf mass per area (LMA) correlates with the ecological performance of plants (Villar *et al.*, 2013). Ferns span quite a large range of LMA-values between 18 and 240 g m^{-2} (Richardson and Walker, 2010). Such a large range can be explained by considerable variation in leaf structure across individual species owing to diverse phylogenetic, biogeographical, and environmental affinities as well as the various techniques used to determine LMA that have been used (selected entire leaves, or parts thereof, with or without median veins, rachis or axis). LMA increases with leaf life-span both in flowering plants and ferns, at least for the temperate understory ferns that have been most studied (Wilson *et al.*, 1999, Karst and Lechowicz, 2007). Tropical ferns are characterized by continuous leaf turnover and their life span may vary from several months to several years (Westoby *et al.*, 2000). There are few data showing a correlation between LMA and leaf longevity for tropical ferns (Mehltreter, 2008, Mehltreter and Sharpe, 2013). The available LMA data for ferns show them to be at the lower end of the range for seed plants (Karst and Lechowicz, 2007). Given its physiological and ecological relevance, several studies (e.g., Niinemets, 1999; Poorter *et al.*, 2009; Puglielli *et al.*, 2015; Villar *et al.*, 2013; Witowski and Lamont, 1991) proposed to break LMA into anatomical components for understanding the traits, which cause LMA variations, as well as physiology and leaf longevity variations. Despite many studies that analyzed the relationship among LMA and net photosynthetic rates, as well as leaf nitrogen and chlorophyll content in ferns (Nishida *et al.*, 2015; Tessier, 2014; Tosens *et al.*, 2015), data about morphological and anatomical traits of ferns and their relationship with LMA are quite limited (Tosens *et al.*, 2015).

The aim of the current research was to (1) determine LMA for evergreen ferns from different families, growing in the same condition, and break down LMA into anatomical components; and (2) analyze the trade-offs between anatomical and morphological leaf traits, which determine LMA variations for three evergreen ferns.

Materials and Methods

Species.—Three ferns largely distributed in Europe (Marchetti, 2001; Pignatti, 1982; Soster, 2001; Tutin *et al.*, 2010) were selected. *Polystichum setiferum* (Forssk.) Moore ex Woynar (Dryopteridaceae) is a rock fern, primarily found in the mountains of southern and western Europe, particularly in shaded deciduous woodlands, lane banks, and often in river-valley woodlands where it prefers slopes and well drained substrates (De Groot *et al.*, 2012; Preston *et al.*, 2002). *Polypodium interjectum* Shivas (Polypodiaceae) occurs mainly in southwest and southern Europe (along the Atlantic and Mediterranean coasts) in moist, shaded habitats, mixed deciduous forests, and on sun-exposed rocks, cliffs, walls, and tree bark (as an epiphyte) (Ivanova, 2006; Marchetti, 2001). *Asplenium scolopendrium* L. (Aspleniaceae) is a European temperate species, occurring in East Asia and North America, growing on limestone substrates in humid, moist habitats, including rocky woodlands, and stream and river banks (Preston *et al.*, 2002; Tutin *et al.*, 2010). All three species are perennials with overwintering leaves, that persistent 12-18 months in the study area.

Study area and climate.—The study was carried out at the Botanical Garden of the Department of Environmental Biology Sapienza University of Rome (41°53′53″ N 12°28′46″ E; 53 m a.s.l.) in January 2015.

The climate of the area is of Mediterranean type. The mean minimum air temperature (T_{min}) of the coldest months (January and February) is 8.6±0.4°C, the mean maximum air temperature (T_{max}) of the hottest months (July and August) is 25.5±0.9°C and the yearly mean air temperature (T_m) is 16.6±6.5°C. The dry period begins in June and ends in August (95.5 mm total rainfall). Total annual rainfall is 866 mm, most of which occurs in autumn and winter (Data from the Meteorological Station of Roma Via Lanciani 38, ARSIAL, Lazio Regional Agency for Development and Agricultural Innovation for the years 2006–2014). All investigated species grew in the area "La Valletta delle Felci" inside the Botanical Garden, that was created in the 1990's. Three representative fertile plants for each species growing under the canopy of *Tilia europaea* L. were selected.

Morphological leaf traits.—Morphological measurements of leaf traits were carried out on four fully expanded fertile shade leaves from each selected plant (n=12). Before measurements, all sori from the abaxial surface of the lamina were removed by a steel needle. Measurements included leaf area (LA, cm^2), leaf length (LL, cm) and leaf width (LW, cm) measured by an Image Analysis System (Delta-T Devices, UK) and leaf dry mass (DM, g), measured after desiccation at 80°C to constant mass. LA and DM of the whole lamina without rachis (for *P. setiferum*) axis (for *P. interjectum*) and massive midrib (for *A. scolopendrium*) were considered. LW was measured at the midpoint of the leaf.

Leaf mass per unit of leaf area (LMA, mg cm^{-2}) was calculated by the ratio between DM and LA (Reich *et al.*, 1992) and leaf tissue density (LTD, mg cm^{-3}) by the ratio between LMA and total leaf thickness (LT, μm) (Wright *et al.*, 2004).

Anatomical leaf traits.—Measurements of anatomical leaf traits were carried out on pinnules (*P. setiferum*), pinnae (*P. interjectum*) or lamina sections (*A. scolopendrium*) from the central part of the fully expanded fresh leaves (three section for each leaves: n=27 sections) and analyzed by light microscopy (Zeiss Axiocam MRc 5 digital camera (Carl Zeiss) using an Image Analysis System (Axiovision AC software). The following parameters were determined in transverse sections: total leaf thickness (μm), abaxial and adaxial cuticle thickness (μm), abaxial and adaxial epidermis thickness (μm), mesophyll tissue thickness (μm); abaxial and adaxial epidermis density (n mm^{-2}), mesophyll tissues density (n mm^{-2}).

Epidermal features were determined from nail varnish impressions (n=27 per species) of the adaxial and abaxial surfaces of the lamina, according to Sack *et al.* (2003). The stomata type was designated following Sen and Hennipman (1981). The following parameters were measured: adaxial epidermal cell length (μm), adaxial epidermal cell width (μm), abaxial epidermal cell length (μm), abaxial epidermal cell width (μm), stomatal cell length (μm), stomatal density (number mm^{-2}).

The fraction of the mesophyll occupied by intercellular air space (f_{ias}, %) was calculated, according to Syvertsen *et al.* (1995) as:

$$fias = 1 - \frac{Am}{lW}$$

where A_m is a cross-sectional area of cells, l — the tissue thickness, and W — the width of the measured section.

Stomatal index (StI, %) was calculated according to Royer (2001) as:

$$StI = \frac{SD}{SD + EabD} \times 100$$

where SD — stomatal density, $EabD$ — abaxial epidermis cell density, where stomata consist of the stomatal pore and two flanking guard cells.

Statistical analysis.—One-Way ANOVA was carried out to test for differences in the measured anatomical parameters between species. Multiple comparisons were done using Tukey's HSD *post hoc* test. Moreover, since LMA is the product of LTD and LT (Niinemets, 1999), ANCOVA models were performed to test for differences in LMA between species with species as the main factor and LTD and LT as covariates. An ANCOVA model was also built to analyze differences in LTD by controlling for differences in LT since the latter was one of the most variable parameters between species and LTD and LT may also be interdependent (Niinemets, 1999). Once the homogeneity of slopes was tested, interspecific differences in the intercepts estimated by the model were evaluated with Tukey's HSD *post hoc* test. Significance threshold was set at $p \leq 0.05$.

Linear regressions were performed to evaluate the relationships between LT and MT (mesophyll thickness), ET (epidermis thickness) and CT (cuticle thickness). All relationships were considered significant at $p < 0.01$ (Wilkinson, 1990).

All statistical analyses were performed using R 3.2.3 (R Development Core Team, 2015).

RESULTS

The three species had different leaf morphology. In particular, *A. scolopendrium* had a simple, entire lamina, *P. interjectum* – a deeply pinnatifid lamina and *P. setiferum* – a bipinnate lamina (Fig. 1). *P. setiferum* showed a higher LL (78.4±10.5), LW (21.6±3.31) and LA (582±144) than the other species (Table 1).

LMA was not significantly different among the considered species (Table 1) varying from 4.28±0.41 mg cm^{-2} (*A. scolopendrium*) to 4.55±0.55 mg cm^{-2} (*P. setiferum*). These values were in the range of other evergreen fern species (Karst and Lechowicz, 2007). In contrast, LTD was significantly different among the species with the highest in *P. setiferum* (278±37.1 mg cm^{-3}) and the lowest in *A. scolopendrium* (127±17.0 mg cm^{-3}), which may be due to anatomical structure of leaf lamina.

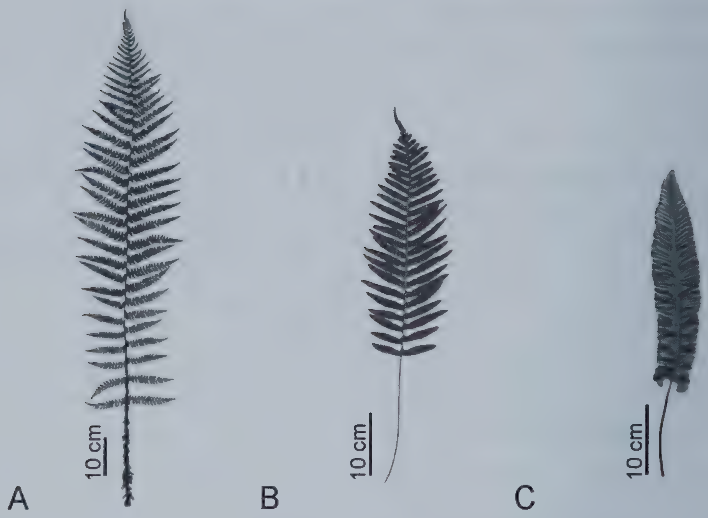

FIG. 1. Leaf morphology of the considered species. (A) *Polystichum setiferum*; (B) *Polypodium interjectum*; (C) *Asplenium scolopendrium*.

The three species had all a typical bifacial leaf anatomy with either polocyte or anomocyte stomates. Adaxial and abaxial epidermis were characterized by one layer of cells with chloroplasts, covered by a cuticle.

The mesophyll of all three species was not clearly differentiated into palisade and spongy parenchyma (Fig. 2), but the cells of the upper rows were arranged more compactly. In particular, *P. setiferum* had the lowest total leaf thickness (163±16.1 μm) (Table 1). The cells of the mesophyll were rotund in the upper part of this tissue and elongated in the lower part forming a reticulated structure. Intercellular air space amounted to 30%. A similar mesophyll structure has been described previously (Bercu and Peterfi, 2001). Cells of the adaxial epidermis (in surface view) were elongated (107±11.8 × 29.5±7.61 μm) with irregularly undulated anticlinal walls. Abaxial epidermis consisted of elongated cells (109±17.9 × 34.7±7.05 μm) with irregularly

TABLE 1. Leaf morphological traits of *Polystichum setiferum*, *Polypodium interjectum*, and *Asplenium scolopendrium*. Mean values (± SE) are shown ($n = 12$ leaves). Different letters indicate significant differences between the species (*ANOVA*, $p < 0.05$).

Morphological traits	*Polystichum setiferum*	*Polypodium interjectum*	*Asplenium scolopendrium*
Type of blade	Bipinnate	Deeply pinnatifid	Simple (entire)
Length (cm)	78.4±10.5 a	55.0±5.43 b	41.0±3.45 c
Width (cm)	21.6±3.31 a	14.3±1.90 b	5.61±0.50 b
Area (cm^2)	582±144 a	223±39.3 b	152±18.5 b
Mass per area (mg cm^{-2})	4.55±0.55 a	4.34±0.47 a	4.28±0.41 a
Tissue density (mg cm^{-3})	278±37.1 a	174±17.1 b	127±17.0 c
Thickness (μm)	163±16.1 a	244±33.8 b	336±32.3 c

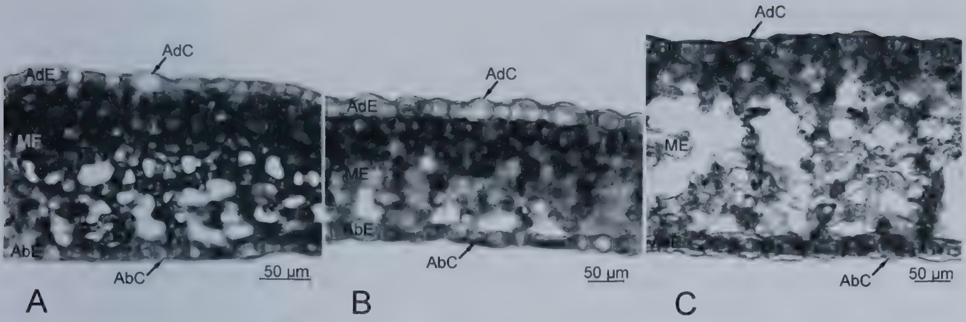

FIG. 2. Leaf cross sections of the considered species. (A) *Polystichum setiferum*; (B) *Polypodium interjectum*; (C) *Asplenium scolopendrium*. AdC – adaxial cuticle; AdE– adaxial epidermis; ME – mesophyll; AbE – abaxial epidermis; AbC – abaxial cuticle.

curved anticlinal walls and stomata. *Polystichum setiferum* had the smallest cell size but the thinnest lamina and highest stomatal density (99.1±13.1 n mm^{-2}).

The *P. interjectum* lamina had a mesophytic organization. The mesophyll consisted of 5–7 rows of mainly rotund cells 187±33.0 µm thick (Fig. 2, Table 2). The fraction of intercellular air spaces (f_{ias}) was 23.2±2.07% in *P. interjectum*, two times lower than in *A. scolopendrium* (Table 2). In surface view, the adaxial epidermis of *P. interjectum* consisted of elongate cells (120±18.0 × 77.5±11.8 µm) with irregularly curved anticlinal walls. The

TABLE 2. Leaf anatomical traits of the considered species. Mean values (± SE) are shown ($n = 27$). Different letters indicate significant differences between the species (*ANOVA*, $p < 0.05$).

	Polystichum setiferum	*Polypodium interjectum*	*Asplenium scolopendrium*
Tissues thickness			
Adaxial cuticle (µm)	4.85±0.58 a	6.90±0.44 b	5.75±0.71 c
Adaxial epidermis (µm)	13.8±2.45 a	25.0±4.31 b	27.4±3.78 b
Mesophyll (µm)	131±14.9 a	187±33.0 b	278±34.9 c
Abaxial epidermis (µm)	10.6±1.70 a	21.4±4.72 b	20.9±3.36 b
Abaxial cuticle (µm)	2.51±0.38 a	5.60±1.25 b	3.77±0.58 c
Tissues density			
Adaxial epidermis, (n mm^{-2})	236±37.0 a	116±47.2 b	58.5±12.0 b
Mesophyll (n mm^{-2})	706±34.6 a	324±47.9 b	159±24.5 c
Abaxial epidermis (n mm^{-2})	328±67.3 a	176±58.2 b	98.9±45.6 c
Intercellular air space (%)	29.9±3.01 a	23.2±2.07 b	41.8±1.61 c
Epidermal features			
Adaxial epidermis cell length (µm)	107±11.8 a	120±18.0 b	88.2±9.28 c
Adaxial epidermis cell width (µm)	29.5±7.61 a	77.5±11.8 b	64.3±10.0 c
Abaxial epidermis cell length (µm)	109±17.9 a	138±24.5 b	109±15.6 a
Abaxial epidermis cell width (µm)	34.7±7.05 a	61.6±11.1 b	50.6±10.1 c
Stomatal cell length (µm)	42.8±2.34 a	60.0±4.18 b	64.8±3.61 c
Stomatal density (n mm^{-2})	99.1±13.1 a	32.5±7.15 b	32.0±3.38 b
Stomatal index (%)	13.7±1.20 b	12.7±2.03 b	9.20±1.27 c

abaxial epidermis had a similar pattern of the anticlinal walls but cells were slightly (13–15 %) longer (138±24.5 × 61.6±11.1 μm) (Table 2). The adaxial cuticle of *P. interjectum* was the thickest of the three species (6.90±0.44 μm). The epidermal cells had an irregular thickness (from 18.1 μm to 34.7 μm). These anatomical traits might be related to the capability of *P. interjectum* to growth in open areas (Ivanova, 2006). *Polypodium interjectum* belongs to the broadly circumscribed, temperate *Polypodium vulgare* complex containing about 17 species (Haufler, Windham, and Rabe, 1995), which is characterized by a high desiccation tolerance (Gildner and Larson, 1992). The irregularly thickened epidermal cells may serve to roll the lamina into a cylindrical tube (Helseth and Fischer, 2005), similar to the bulliform cells on the upper surface of the leaves of many grasses. The relatively thick cuticle limits transpiration.

Asplenium scolopendrium had the greatest total lamina thickness (336±32.3 μm). The mesophyll consisted of loosely placed elongated cells with large air spaces between them (f_{ias}=41.8±1.61 %). In surface view, cells of the adaxial epidermis were isodiametric (88.2±9.28 × 64.3±10.0 μm) with U- to Omega-like undulation patterns of the anticlinal walls. The abaxial epidermis in surface view consisted of elongated cells (109±15.6 × 50.6±10.1 μm) with irregularly curved anticlinal walls and stomata (Table 2). *Asplenium scolopendrium* had the lowest StI (9.20±1.27%), which might be correlated with ability of this species to grow in moist habitats (Ticha, 1982).

On the whole, the results highlight that LMA was not significantly different among the three species, despite the differences in the anatomical structure of the lamina.

Discussion

LMA is a complex characteristic that varies significantly and depends on the plant species, their functional groups and different habitats (Poorter *et al.*, 2009). Using plants from Botanical Garden might negate the effects of different environmental and climatic factors such as soil type or microclimate on morphological and anatomical characteristics of plants.

As was estimated early, LMA value reflects variation in quantitative anatomy, such as the thickness of the cuticle, epidermis, mesophyll, and other cell layers (Niinemets, 1999). Higher LMA in woody evergreen species is mainly due to a greater leaf thickness, caused by a larger volume of mesophyll and air spaces (Villar *et al.*, 2013). Despite similar LMA values, the lamina thickness was significantly different (Tab. 1). The three species can be placed into a series of increasing the lamina thickness: *P. setiferum* → *P. interjectum* (37% more) → *A. scolopendrium* (106% more than *P. setiferum*). This increase was mainly due to the increase of the mesophyll fraction. The absolute thickness of the mesophyll fraction increased in the same way, maintaining, however, about the same percentage (76-82%) among all leaf fractions.

Because LMA is the product of leaf thickness and leaf density (Villar *et al.*, 2013), the reduction in leaf thickness resulted from the small size of cells and

TABLE 3. Common slope and intercept values (±SE) estimated for each ANCOVA model run for the considered species. In the upper and intermediate panels leaf mass area (LMA) is taken as response variable and leaf tissue density (LTD) and total leaf thickness (LT) as covariates, respectively. In the lowest panel LTD is taken as response variable and LT as covariate. All the models were run with species as main effect. Bold values refer to the significance of the covariate. Different superscript lower-case letters indicate significant differences between intercepts assessed by Tukey's HSD post hoc test. Significance threshold was set at $p \leq 0.05$. More details on the overall significance of the ANCOVA models as well as on common slope and intercept tests are shown in Fig. 3.

	LMA ~ LTD	
Main effect	Common slope	Intercept
Polystichum setiferum	0.012382 (0.002472)	1.112398 (0.400975)[a]
Polypodium interjectum		2.186445 (0.188315)[b]
Asplenium scolopendrum		2.706554 (0.331112)[c]
	LMA ~ LT	
Polystichum setiferum	40.8516 (31.5457)	3.8788 (0.5847)[a]
Polypodium interjectum		3.3173 (0.3404)[a]
Asplenium scolopendrum		2.8922 (1.0801)[a]
	LTD ~ LT	
Polystichum setiferum	4271.304 (1559.412)	348.091 (28.901)[a]
Polypodium interjectum		281.15 (16.826)[b]
Asplenium scolopendrum		272.113 (53.390)[b]

the increase in tissue density. Thus, *P. setiferum,* which had the smallest leaf thickness, is characterized by the largest values of tissue density (Tab. 2).

The relationships among the investigated leaf traits highlight that variations in LMA are the result of variations in LTD. On the contrary, LT does not scale significantly with LMA (Table 3, Fig. 3), increasing with MT (Table 4). An increase in mesophyll thickness seems to be related to a greater cell wall area for CO_2 diffusion, which decreases the liquid-phase resistance. On the other hand, an increased LT tends to increase the path length from stomata to cell wall surfaces increasing gaseous diffusion resistance (Niinemets, 1999). This can be avoided by increasing f_{ias} with an LT increase resulting in a LTD decrease, according to Witkowski and Lamont (1991) and Puglielli *et al.* (2015).

TABLE 4. Linear regression analysis between total leaf thickness (LT) and mesophyll thickness (MT), epidermis thickness (ET) and cuticle thickness (CT) for the considered species. Slope, R^2 and p value per each relationship and species are shown. The relationship were considered significant (bold p values) at $p<0.01$.

	Polystichum setiferum			*Polypodium interjectum*			*Asplenium scolopendrium*		
LT vs	Slope	R^2	p value	Slope	R^2	p value	Slope	R^2	p value
MT	0.0001934	0.71930	0.0003124	0.0001310	0.6446	0.001223	0.0001462	0.7243	0.000265
ET	0.0004899	0.5996	0.001888	-0.00009951	0.0359	0.5552	-0.0005066	0.3139	0.03389
CT	-0.000637	0.034	0.5661	0.0006407	0.1065	0.1595	0.002438	0.1853	0.09083

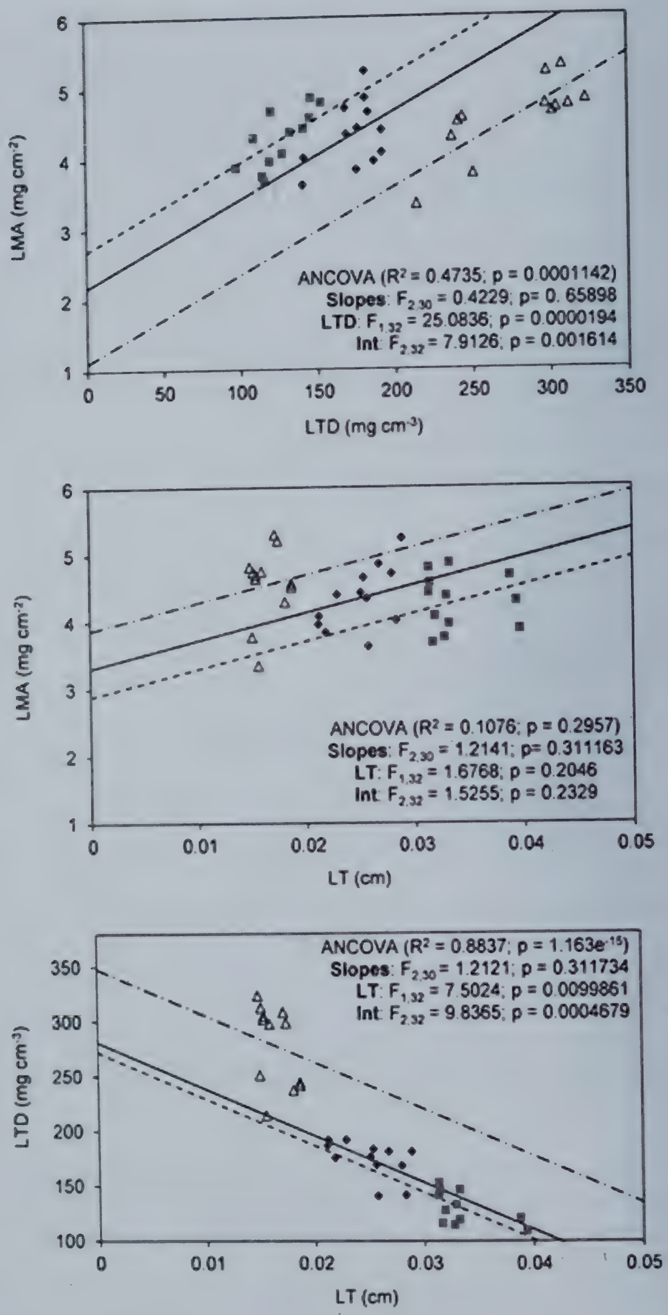

FIG. 3. Linear regression analysis between. (A) leaf mass per area (LMA) and leaf tissue density (LTD); (B) leaf mass per area (LMA) and total leaf thickness (LT); (C) leaf tissue density (LTD) and total leaf thickness (LT) for *Polystichum setiferum* (white symbols), *Polypodium interjectum* (black symbols) and *Asplenium scolopendrium* (gray symbols). Individual measurements were used as experimental units (ANCOVA, $n = 20$, $p \leq 0.05$). Common slope and intercept tests as well as the effect of the covariate are also shown. Regression lines were drawn by using the parameters shown in Table 3.

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Published on 28 February 2017

SHORTER NOTES

FIRST RECORD OF THE AMERICAN NATIVE FERN *THELYPTERIS KUNTHII* (DESV.) C. V. MORTON FROM EUROPE.—*Thelypteris kunthii* is a fern with known native distribution from the southeastern USA into Mexico, Central America, the Antilles, and sparingly in northern South America. In addition, it is weakly naturalized in a few other places of the New World (A.R. Smith pers. comm.), but not previously reported outside of the Americas. This fern is considered semi-weedy species where it is native. It usually grows along roadsides, in ditches, on riverbanks, in woodlands and in limestone sinks (Smith, Univ. Calif. Publ. Bot. 59:1–143. 1971).

In 2009, I found a population of an unknown Thelypteridaceae species along the Guadalquivir River as it passes through the town of Cordoba, in southern Spain. Subsequently, flooding hid this population; however, I recently noticed plants of this species at the same locality, and collected a specimen, which was identified by Alan R. Smith as *Thelypteris kunthii*:

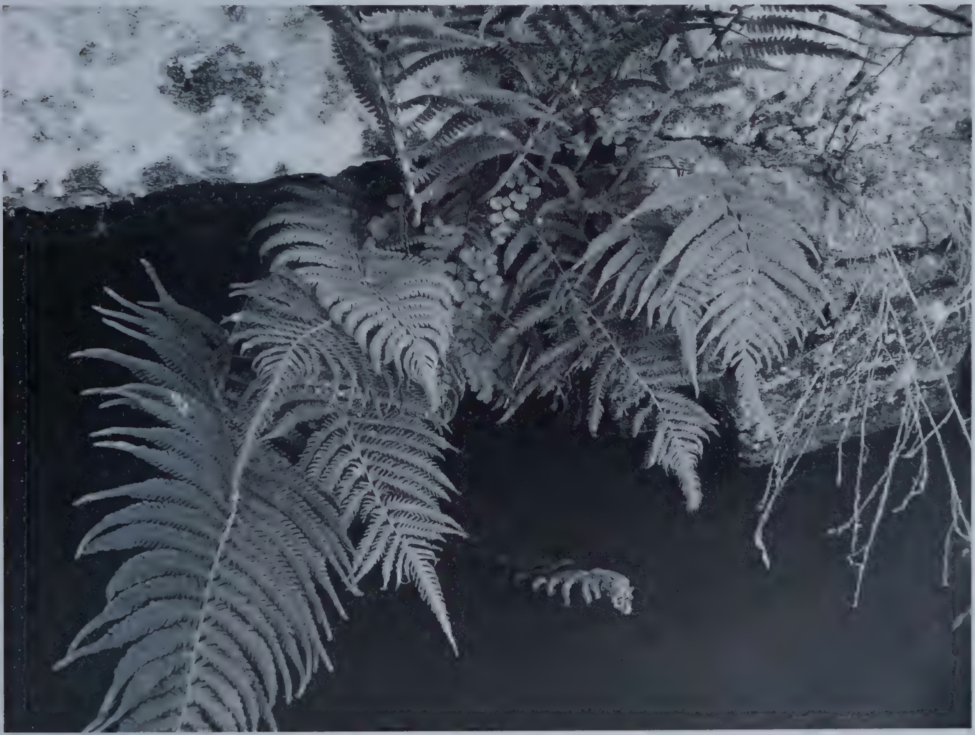

FIG. 1.—*Thelypteris kunthii* in the new European population.

Thelypteris kunthii (Desv.) C. V. Morton, SPAIN: CORDOBA: 30SUG4494, Cordoba, Molino de Martos, 96 m, 13.VII.2016, *J. López Tirado s.n.* (COFC 62615).

The population grows in crevices in an ancient watermill (Fig. 1), alongside *Adiantum capillus-veneris* L. and *Nephrolepis exaltata* (L.) Schott. This fern is widely used as ornamental. Also in Cordoba it is cultivated in private houses and disseminated by stem division. Moreover, I noticed that it reproduces by spores. This population is in an area difficult to access, thus it has not been planted. Since 2009, this population has doubled the specimens. Nowadays it comprises about ten plants, some of them fertile, therefore *Thelypteris kunthii* can be considered as naturalized.

Based on the online European floras consulted (www.anthos.es, flora-on.pt, www.gbif.org, www.actaplantarum.org; last access August 2016) as well as *Flora Europaea* (Valentine and Moore, *in* Flora Europaea. Cambridge University Press. 1:17–18. 1993), this appears to be the first record of *Thelypteris kunthii* naturalized in Europe. There are other cases of ornamental fern species escaped from cultivation, like *Nephrolepis exaltata*. This fern has been recently recorded in the nearby province of Seville (Jiménez-Mejías *et al.*, Lagascalia 30:476. 2010) and detected now for the first time in the province of Cordoba. Monitoring the current population and other potential areas, such as other watermills, can lead to a good knowledge about invasiveness of these non-native ferns in Europe.

The author is very grateful to Alan R. Smith, University of California, Berkeley (UC), who identified this fern and made comments on a draft of this manuscript. This is a contribution from the CEIMAR Journal Series.—JAVIER LÓPEZ-TIRADO, Department of Integrated Sciences, International Campus of Excellence of the Sea (CEIMAR), University of Huelva, s/n Tres de Marzo Avenue, Huelva, Spain, 21071, email: javier.lopez@dbasp.uhu.es

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SHORTER NOTES

CYTOLOGICAL CONFIRMATION OF TETRAPLOID *ASPLENIUM CETERACH* IN IRAN.—*Asplenium* L. (Aspleniaceae) is a subcosmopolitan homosporous fern genus with at least 700 species (Pteridophyte Phylogeny Group. Journal of Systematics and Evolution 54: 563–603, 2016). Nine species of *Asplenium* have been recorded from Iran (Khoshravesh, R., H. Akhani, M. Eskandari, and W. Greuter. Rostaniha 10:1–129. 2009), but their ploidy levels in the country have not been documented. This paper reports for the first time the ploidy level of *A. ceterach* L. from Iran.

Asplenium javorkeanum Vida, the diploid ancestor of *A. ceterach*, is known from Albania, Bulgaria, Croatia, Greece, Hungary, Italy, Romania, and Slovenia (Vida, G. Academy of Science Hungarica 9:197–215. 1963; Reichstein, T. Botanica Helvetica 91:89–139.1981). The small and epilithic *A. ceterach*, an autotetraploid derivative of *A. javorkeanum*, has a wider distribution and is recorded throughout Europe (The Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo. 1972), southwest Asia, western Himalayas, and in Africa in Eritrea and Somalia (Viane, R. L. L., H. Rasbach, K. Rasbach, and T. Reichstein. Bocconea 5:279–300. 1996), the Arabian Peninsula (Collenette, S. MEPA, Flora Publication 1. 1985) and Yemen (Christ, H, Buchdruckerei K. J. Wyss, Bern. 1900). In Asia, it has been collected from different countries including Afghanistan, China, India, Pakistan, Siberia, Tibet, and Turkey (Van den heede, C. J., S. Pajarón, E. Pangua and R. L. L. Viane. American Fern Journal 94:81–111. 2004). In Africa, *A. ceterach* is known from Algeria (Jahandiez, E. and R. Maire. Minerva, Alger, Algeria. 1931; Van den heede *et al.* 2002), Egypt, Libya, and Tunisia (Maire, R. Volume I. Pteridophyta- Gymnospermae- Monocotyledonae: Pandanales, Fluviales, Glumiflorae. Paul Lechevalier, Paris. 1952; Lovis, L. D. Advances in Botanical Research.4:229–415. 1977); and in Cyprus (Van den heede *et al.* 2002).

Distinguishing characteristics of *A. ceterach* include dense covering of brownish ovate-lanceolate scales on abaxial leaf surfaces and anastomosing venation (Lin, Y. X. and R. Viane. Aspleniaceae. Pp. 267–316, *in* Z. Y. WU, P. H. Raven & D. Y. Hong (eds.), Flora of China, Vol. 2–3 (Pteridophytes). 2013). The base chromosome number for *Asplenium* is x=36 (Wagner, W. H. Evolution 7:103–118. 1954) and there are five levels of ploidy including diploids, triploids, tetraploids, pentaploids, and hexaploids. The diploid *A. javorkeanum* has been known from central Eastern Europe, Italy, Greece, and Turkey. The triploid *A.× mantoniae* with n=108 has been reported from Eastern Mediterranean islands, Southern Europe, China, and Afghanistan, which is the region nearest to the current study site. A few records of pentaploids and hexaploids also occur in Mediterranean Europe (Vida, G. Acta Botanica Academy of Science Hungarica 9:197–215. 1963; Viane, R., A. C.

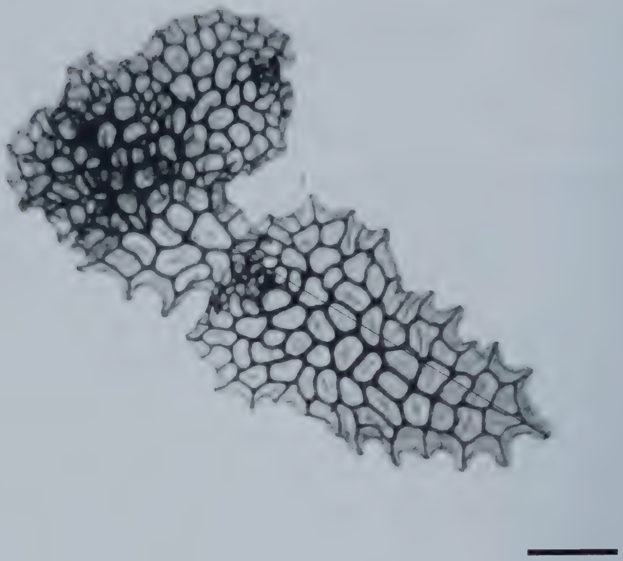

FIG. 1. Scales with mostly pentagonal or hexagonal cells. Scale bar= 20 μm.

Jermy and J. D. Lovis, *Asplenium*. Pp. 18-23, *in* T. G. Tutin, N. Z. Burges, and A. O. Chater, eds. Flora Europeae, Vol. 1. 2nd ed. 1993; Pintér *et al.*, Organisms Diversity Evolution 2:299–311. 2002; Van den heede, C.J., S. Pajarón, E. Pangua, and R. L. L. Viane. American Fern Journal 94:81–111. 2004).

Thirteen individuals belonging to eight vegetative patches with an average surface area of ca 600 cm^2 were collected from their natural habitat in the Chelmir valley (Lat.: 37°23′ 42.5" N; Long.: 58°52′ 00" E; 1030–1100 m; Coll.: L. Ghoreishi), Tandoureh National Park, NE Iran on 30 May 2014. The patches were observed in the cracks of calcareous rocks in shaded environments. Voucher specimens were deposited at IAUM. For identification we used Khoshravesh *et al.* (2009), as well as comparison to identified samples in FUMH. Specimen characteristics were consistent with those of *A. ceterach* and had abaxial leaf surfaces densely covered with scales that averaged 2.7 × 1.3 μm, and contained cells with tetra- to hexagonal shape (Fig. 1). Each sorus contained 18–28 sporangia with mean diameter of 120 μm, which bore an annulus containing 17–22 cells. For leaves 6–7 cm long, the mean number of sori was 136. The length of spores as measured in distilled water was 39.8–41.1 micrometers. The spores had an ornamented perispore.

Chromosomes were observed in spore mother cells following the squash technique of Windham, M. and G. Yatskievych (American Journal of Botany 90:1788–1800. 2003). To increase the chance of observing meiotic metaphase chromosomes, pinnae of living plants were collected every half an hour from 9:30 am to 3 pm and were pretreated with a saturated solution of paradichlorobenzene. Pinnae were kept on ice during collecting for a

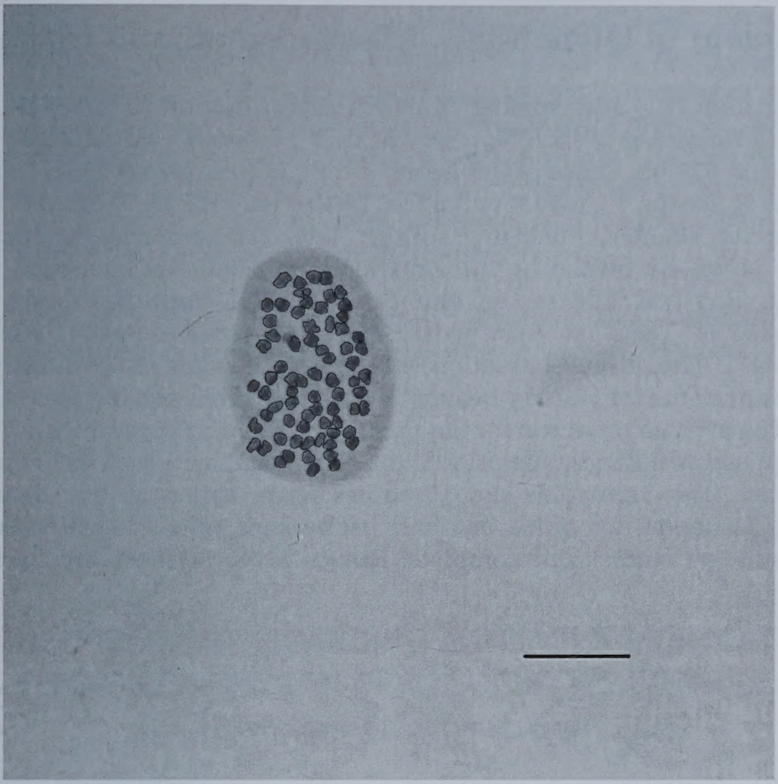

FIG. 2. Chromosome preparation from *A. ceterach* 72 II, 4*x* and explanatory diagram. Scale bars= 50 μm.

maximum of 6 hours and then fixed in Carnoy's Solution: ethanol: glacial acetic acid: chloroform (6: 1: 3). Sporangia were removed from the fixed leaves and squashed in acetocarmine. Photographs were obtained using a digital camera OLYMPUS DP71 connected to an OLYMPUS BX51 microscope.

We observed meiotic metaphase divisions in pretreated samples collected at 10:30 and 11 am. In material collected at other times, prophase, anaphase, and young spores were mostly seen. The chromosome number was 72 bivalents (n= 72 $^{\mathrm{II}}$) (Fig.2).

Although previously recorded from Iran based on morphological characters (Parsa, A. and Z. Maleki, Ministry of Science & Higher Education of Iran, Tehran. 1978; Khoshravesh *et al.* 2009), ours is the first confirmation that the Iranian plants are tetraploid.

We thank Mr. M. R. Joharchi, the curator of FUMH for access to the herbarium material.—LIDA GHOREISHI AND MAHROO HAJI MONIRI, Department of Biology, Faculty of Sciences, Mashhad Branch, Islamic Azad University, Rahnamaie str., Postal Code: 917 56 87 119, Mashhad, Iran, email: m.h. moniri@mshdiau.ac.ir

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